

Rigging the price of silicon chips

The agreement signed last week between Japan and the United States will not in the long run keep Silicon Valley healthy, and meanwhile will be a recipe for trouble.

ONLY a few days after one of the largest steel-makers in the United States, the conglomerate LTV, ran for the shelter of the US bankruptcy laws, trade officials in Washington were foolishly celebrating last week the agreement they have negotiated with their opposite numbers in Japan to regulate the sale of semiconductor memory chips between their manufacturers. The coincidence is striking and also chilling. LTV has been driven into queer street by the long decline in the demand for steel when the world is swimming with the capacity to produce the stuff. During much of that time, the US government has done what it can to protect the domestic industry from overseas competition by a variety of restrictions on the free import of steel. Voluntary quotas have been negotiated with European steel-makers, while the policy of protection has been stiffened by the usual array of anti-dumping suits in the courts. The result is that the US steel industry has been kept alive, but only just.

Technically, the industry has limped along, maintaining existing plants without the funds to improve them to a point at which they might compete effectively with steel-makers in Japan and Korea. The companies involved have in the process been locked into a declining business when their long-term interests would have dictated diversification. What has happened to the US steel industry is yet another proof that, in a competitive world, protection is more often the kiss of death than a safeguard. How soon will this truth be demonstrated in the manufacture of silicon chips?

The new agreement is believed to cover something like 90 per cent of the semiconductor manufactures supplied by Japan to the United States. All the bread and butter in the trade will be regulated, but, almost by definition, the regulations will not apply to the most advanced components, those so novel that competition has not yet developed and whose lucky manufacturers can charge what prices they choose, not to mention the chips not yet invented. The agreement binds both signatories to ensure that chips are not sold for export at prices other than those defined as fair, which implies that manufacturers will have to recover in their prices not merely the marginal cost of production but also a contribution towards the capital costs of the plants in which chips are made.

Technically, the agreement is a recipe for endless bickering. Difficulties have arisen about the prices charged for chips because of the huge difference, in a capital-intensive business, between the marginal cost of production and that which fully amortizes the cost of the equipment with which chips are made. Who will now decide what production overheads are fair? But in practice, the effect of the agreement promises to be unambiguous: prices of chips of all kinds will be increased for everybody, often substantially.

For a time, no doubt, there will be rejoicing in Silicon Valley, the Californian hub of the US computer industry. Small companies whose backs have been against the wall for months on end will be able to walk tall again, sheltered from the formidable competition of Japanese manufacturers by increased prices. The new deal may not restore the full excitement of the 1970s, when for a time it seemed as if Californian entrepreneurs had stumbled on the modern equivalent of the philosophers'

stone, a way of turning silicon into gold. But there will be fewer lawyers preoccupied with the intricacies of Chapter XI, the provisions of the bankruptcy laws that allow a company to keep trading while protected from the most pressing claims of its creditors. Undoubtedly there will be a spell of months and possibly years, during which the sales of the US chip-makers will increase, both in volume and in profitability.

Japanese manufacturers will be compelled to compete in other ways, two of which are easily foreseen. First, they will pay even more attention than is their present custom to the quality of what they manufacture and put on the market. Second, compelled by the agreement to charge more for what they sell at present and thus made even more profitable than they are now, they will invest even more enthusiastically in novel semiconductor devices. A few years from now, Silicon Valley will probably still be a high-cost producer, but may well have lost its present reputation for daring design behind the shield for foreign competition now being provided. That is what has happened to steel manufacture in the United States.

Meanwhile, the economic cost of the new agreement will be felt throughout the world. To prevent manufacturers in Japan selling cheaply into the United States through the intermediary of third countries, price-fixing will apply to chips sold by both Japanese and US companies in, for example, Europe. One result will be that the prices of electronic equipment will be increased. Another will be that European chip-makers will enjoy the benefits being showered on Silicon Valley at a crucial time in their strategic history, when the case for making chips indigenously is stronger than it has ever been. Whether European companies will ever be able to match those in Japan in quality and cost is an open question, but now at least they will have an opportunity to do so. Is that what the United States intends?

That the new agreement will not serve its intended purpose, but others that cannot accurately be foreseen, is beyond dispute. Yet one malign side-effect is easily predicted. To the extent that the new agreement on chips establishes an inter-governmental cartel for the regulation of prices in international markets, it will be an awkward precedent that is bound to come to haunt the United States, hitherto an admirably staunch supporter of the doctrine that free trade is the only effective means of arriving at an economic division of labour in the world. Which other governments, in respect of other manufacturers, will now be tempted to follow suit? All they will have to dissuade them is the example of the Organization of Petroleum Exporting Countries (OPEC), now in collapse along with the price of crude oil. □

Selling the family silver

Turning public into private enterprise is fashionable, but not best done by public servants.

LORD Stockton, previously Mr Harold Macmillan, last year startled the British government which he supports politically by reproving it for "selling the family silver" in order better to balance its accounts. The circumstances are now familiar, and by



no means exclusively British, even if the present British government is among the most zealous of those attempting to turn public into private enterprises. (M. Chirac's government in Paris seems anxious to follow suit, if President Mitterrand's reluctance is in the end overridden by the French Assembly.) Already a majority of the shares in the previously nationalized telecommunications network and the aerospace company have been sold to the public. The gas monopoly is to be sold later in the year, but there will be a long delay before the complicated water industry can be made private, and it remains to be seen whether the government can find a buyer for the motor-car manufacturer it owns who will be acceptable to its own supporters (who, with others, killed the attempt to sell part of it to US General Motors a few months ago).

Meanwhile (see p.491), at the other end of the spectrum financially, the government has managed to work out a way of selling off its interest in the breeding of commercial crops through the Plant Breeding Institute at Cambridge (England). In principle, this is a sensible step to take. Like all the other research institutes operated by the Agricultural and Food Research Council (AFRC), the Plant Breeding Institute has had a rough and over-spartan time in the past few years. Budgets have been held stagnant or even cut, with the result that vacant posts have not been filled even when there is productive work for their potential occupants to do. But the institute differs from most others in that the benefits of the work it does are translated directly and tangibly into monetary terms. The new varieties developed at the institute are sold to farmers, in Britain and overseas, through bodies such as the National Seed Development Organization, which regularly makes a substantial profit on its operation that, under the present system, has been dutifully turned over to the British Treasury. Taken together and averaged over the years, the profits of the seed organizations are not very different from the costs of the research that has sustained them, which vividly illustrates the folly of having kept the institute on such short commons all these years. Now the breeding and marketing organizations are to be put up for sale as a package. There is a chance that the new entity will be able to make its way in the world relatively free from worry, and even more conscious of how its skills (widely acknowledged to be high) should be most effectively deployed.

That is the plus side of the account. The other is the decision that, in the process of privatization, the Plant Breeding Institute should be split in two. Plant breeding pure and simple (if there is such a thing these days) is part of the commercial package, but molecular biology will stay in the public sector. That, at least, is the intention, however little sense it makes when plant breeders the world over are bent on using genetic manipulation to give crop varieties characteristics they would not acquire naturally. If the institute is indeed sold off successfully (as seems likely), the new owners would promptly set out to recruit a team of molecular biologists to make up for the loss now being decreed. They would not have far to look.

None of this implies that research councils such as AFRC should withdraw from the molecular biology of plants. On the contrary, there is the strongest possible reason why the scale of effort in this field should be increased even in circumstances such as those in Britain, where farming is no longer strictly an economic activity (see *Nature* 322, 195; 1986). But the opportunity for spinning off a substantial team of people working in the field already should be regarded as a heaven-sent chance for creating another. This is a chance to sell the family silver and to get another set. Luckily, it is not too late for the government and AFRC to change their minds. Indeed, on the principle that the British government in its present mood is unlikely to increase the funds available for supporting basic research but, equally, is unlikely to have the gall to cut them even further, there is a strong case for scouring government departments for other parts of the public research enterprise that might be thrown to the commercial wolves. □

Taxes make big waves

The effects of US tax reform will be felt far outside the United States.

BEFORE the summer is out, the US Congress will have put the finishing touches to the tax bill that is almost certain to be what the Reagan administration is remembered for. That, at least, is the plan, which is likely to be sustained by the way in which general enthusiasm for lower percentage rates of tax promises to overcome taxpayers' attachment to the labyrinthine network of deductions from income they have long regarded as their birth-right. While the marginal rates of tax are still to be determined, the mere idea that there may be just two, at 15 per cent and 27 per cent, will excite the envy of taxpayers elsewhere, many of whom are used to surrendering more than half of the extra they earn. Even if the rates at which optimistic congressmen are aiming cannot be achieved, or if it turns out that the extra taxes that business will have to pay are economically damaging and must be reduced, the simplicity of the new tax bill cannot fail to be seductive elsewhere.

There will be more immediate and specific consequences, one of which may benefit the economic partners of the United States. The details of the new bill are still being negotiated between the US Senate and the House of Representatives, and there is much to be worked out. The Senate version of the bill is generally considered friendlier to business, perhaps reflecting its Republican majority. By contrast, the House version generally provides greater savings to individual taxpayers. Arriving at a compromise that will be acceptable to at least a majority of legislators is proving to be at least as hard as analysts predicted it would be. But one feature likely to emerge intact is the proposed abolition of the tax shelter embodied in the device of the limited partnership; its effect will be to send venture capital scurrying to other places in the industrial world where investment in innovation is still encouraged by such means. The principle is that people may invest part of their income in some new venture, which may be a new company or even just a new project on which a company is engaged, pay no tax on that slice of income but pay capital gains tax on their profits if the venture should succeed. The device has been widely used in the past few years in, for example, the development of biotechnology. If, now, such opportunities are no longer available in the United States, individuals and corporations with income arising elsewhere will be tempted to look for them where they can. Already there are people in Europe calculating that the flow of venture capital across the Atlantic may be substantial.

The other side of that coin is that the dazzling attractions of the tax reform bill may, over a longer period of time, work in the other direction. Ever since the Second World War, the United States has been a magnet for technical people elsewhere. The canard that engineers in the United States are so busy at their profession that they have no time for teaching, with the result that the education of young engineers is in the hands of people from overseas, is at least a half-truth. Other governments are often alarmed enough to mount formal inquiries into what they call the brain drain, only to discover that their emigrating technical people proclaim that they are leaving for the United States only because working facilities are better there, or that they seek the chance of working alongside particular colleagues. But this is humbug, and should be recognized as such. Is there any reason to believe that technical people are immune from the prosperity that makes the United States attractive not merely to other professionals but to the army of unskilled people who seek the same haven? By that test, the rate at which technical people depart for the United States will only be accelerated when the tax bill passes. Are governments elsewhere reconciled to that alarming prospect? And will they choose to follow the only course by which they might convincingly respond, that of mimicking what is now happening in Washington? □

US budget

Winners and losers worry about Gramm-Rudman

Washington

CLEAR signs emerged last week that the US Congress may be unwilling to support some of the increases in research funds proposed in President Reagan's budget for the next financial year, beginning in October. Faced with strong political pressure to reduce the federal budget deficit, the Appropriations Committee of the House of Representatives recommended providing the National Science Foundation (NSF) with \$136 million less than the \$1.686 billion sought by the President, giving only a slight real increase over this year's level. A small cut of \$44 million from the President's request was also proposed for the National Aeronautics and Space Administration (NASA).

The still uncertain effect of last year's Gramm-Rudman deficit control act makes prediction of agencies' budgets difficult. But academic and scientific bodies did not hide their dismay last week that Congress had failed to support the 8 per cent increase sought by the President for NSF, the main federal source of support for non-military basic research. Jack Crowley of the Association of American Universities was disappointed that a key committee of Congress saw increased support for basic research as "an expendable priority". Others pointed out, however, that the committee had in the same legislation approved significant reductions for other agencies and that it had gone as far as it could to protect NSF.

The National Institutes of Health (NIH) are, however, another story. The House of Representatives excelled even its own customary generosity to NIH by voting to provide \$6,153 million for the institutes, \$1,073 million more than the President's budget request and \$893 million more than the 1986 figure. The House specifically noted that it wanted to see 6,200 new extramural research grants next fiscal year, about 700 more than the administration wanted. While the Senate might temper the House's enthusiasm, there seems little doubt that Congress's strong support for NIH is unchanged from previous years.

During the House debate that preceded the vote, much was made of the growing death toll due to AIDS (acquired immune deficiency syndrome), and \$336 million was voted for efforts to combat the disease, \$133 million more than the President had requested.

Other appropriations bills on which the House has taken action include a bill providing funds for the Department of

Energy. Here the House has also followed the pattern of previous years by voting to restore some of the large cuts proposed by the President in solar power and magnetic fusion research. But at the same time it cut \$536 million from the \$8.330 billion budget request for the department's defence programmes, to take account of limits placed by Congress on growth of the Strategic Defense Initiative.

The House has, however, alarmed academic groups by allowing to pass in the same bill \$69.7 million for research and construction projects at eight named institutions that have not been through the usual process of authorization by Congress and have not been subject to peer review. Most scientific and academic bodies are opposed to Congress's growing tendency to provide funds for specific unreviewed construction projects from research budgets. But Congress seems in no mood to compromise. An amendment to delete funds for the unreviewed projects was defeated by 315 to 106. The Senate has yet to consider the matter, but recently defeated a move to delete funds in a Department of Defense emergency money bill for unreviewed projects at nine universities (see *Nature* 322, 4; 1986).

Most of the appropriations now at various stages of approval in the House have not reached the Senate. But the exact amounts specified by both houses could become largely irrelevant if, as some expect, a legislative formula is found to allow automatic cuts to be made to agencies' budgets to make them comply with the \$144,000 million budget deficit target specified by Gramm-Rudman.

Although the mechanism specified in Gramm-Rudman for making the automatic cuts was ruled unconstitutional by the Supreme Court in July, efforts are under way to modify it. The court's objection was over the pivotal role played by the General Accounting Office in determining the size of the cuts; one proposal now under consideration would meet that difficulty by substituting the executive branch's Office of Management and Budget.

A preliminary estimate of the likely budget deficit next year will be made on 15 August and, if the target is exceeded by more than \$10,000 million, the necessary across-the-board cuts would then be calculated. If Congress has not by then completed appropriations for the next fiscal year, the 1986 levels will be used in the calculation, a prospect particularly alarming for agencies such as NSF that had been

in line for large increases. Even if the automatic cuts are not made, however, there will be strong pressure on Congress to meet the targets in the Gramm-Rudman law.

Many congressional staff now consider it inevitable that the cuts will be triggered: slow economic growth has reduced expected revenues and a deficit of around \$220,000 million seems likely, if Congress sticks to the budget guidelines agreed earlier. The cuts would probably be much bigger than the 4.3 per cent made this year. But congressional elections in November could prove to be the wild card. Some observers think that Congress will yet devise some way of getting itself off the Gramm-Rudman hook before November.

Tim Beardsley

Inventors' rewards

THIRTY-three British universities and colleges have gained permission to exploit financially inventions arising from research funded by the research councils. Until now the British Technology Group (BTG) has had right of first refusal for the commercial development of government-funded research, a process that its just released annual report shows is not without its dangers.

Last year, BTG, a state-owned management company formed to assist the commercialization of innovative ideas, had to spend more than a million pounds defending its patent rights. Most of the money went on fighting for the hovercraft — a great British invention back in 1959 now being put into use by the US Armed Forces. The trouble is they are not paying the royalties and "American lawyers are very expensive" as a BTG spokesperson put it. But BTG still managed an income of nearly £20 million from licensing and industrial projects.

Before being granted permission to try for similar profits on their own, the universities had to assure a scrutiny committee that they knew what they were about. The chief aim, in line with government policy, is to provide new incentives for individual researchers as well as their universities. That aim can be satisfied only by arrangements in which inventions with a potential for commercial exploitation can be spotted early; flexible routes for their exploitation, including setting up new companies and forming partnerships with industry, can be quickly set up; and inventors properly rewarded. That may mean the inventor will get all the proceeds if the invention has a low level of return, and the university gets a cut when bigger profits can be made.

Universities that have not yet received permission to exploit their inventions are likely to do so in the near future. Some, though, will continue to use existing arrangements with BTG.

Alun Anderson

European nuclear power

Muddle over safety measures

A SWINGING attack on the European Economic Community (EEC)'s nuclear safety and public information record was delivered in Brussels last week by its commissioner for the environment, Stanley Clinton Davies. His remarks were apparently based on a new report from an expert committee — but those involved in the report's preparation claim it reaches wholly opposite conclusions.

The report, due to be published in a few months, was drawn up by those close to the nuclear industry and it supports its policies, according to Commission sources. It is regarded, however, only as advice and the European Commission is free to draw its own, even contradictory, conclusions.

Clinton Davies's interpretation was certainly affected by the uncoordinated response by European states to the Chernobyl nuclear disaster, which came late in the expert group's year-long deliberations. In that context, the commissioner felt it necessary to criticize the poor and late provision of public information in some countries (by implication, France), and the very disparate actions taken in response to contamination. "Everyone got under their own umbrella", said a Commission spokesman, and some umbrellas were big and others patchy.

The environment commissioner's objective in attacking national safety measures is thus clear: he believes there is a central coordinating role to be played by the European Commission in matters of setting nuclear safety levels and the rapid collection and dissemination of information across national boundaries in the case of accidents. Thus Clinton Davies last week attacked, by implication, West Germany, Italy, the Netherlands, Belgium and others for failing to implement an EEC directive on "basic norms" for

Made in Japan label on launchers

Tokyo

NEXT week Japan takes its first big step towards independence from US rocket technology with the test launch of its new H-I rocket.

Throughout its 17-year history, the National Space Development Agency (NASDA) has depended on licensed US rocket technology to boost its satellites into orbit. Breaking with that tradition, the H-I has a domestically developed second-stage cryogenic engine, the LE-5. On its first test flight, a two-stage version will be flown; later flights will be of the full three-stage rocket. And, unlike its predecessors, the N-I and N-II, the rocket will also have a new Japanese-built inertial guidance system.

Scheduled to blast off on 13 August, the H-I will carry three inexpensive payloads into orbit: the Experimental Geodetic Payload (EGP), a hollow globe over two metres in diameter, covered in mirrors and laser reflectors, that will be used by the Maritime Safety Agency and the Geographical

Survey Institute for laser triangulation surveys; the Japan Amateur Satellite-1 (JAS-1) for ham radio enthusiasts; and a magnetic bearing flywheel to be tested in space before incorporation into future satellites. The total budget for the launch including the satellites is 16,000 million yen (about \$100 million).

From 1988 the rocket is planned to go into regular service placing communications, broadcast, meteorological and Earth resources satellites into geosynchronous orbit. But with a maximum payload capacity of only 550 kg, the H-I is still small by international standards, and by 1992 NASDA hopes to replace it with the H-II, a two-stage launcher, capable of boosting a 2,000 kg satellite into a geostationary orbit, that will be 100 per cent "made in Japan".

Although still not in the big space league, the H-II will allow Japan to compete in the international market for the launch of small to medium scale satellites in the 1990s.

David Swinbanks

emissions and radiation exposure, which had been due for ratification last April. Europe's principal nuclear states, Britain and France, are not in default on "basic norms", but according to a spokesman "most" of the 12 EEC nations have failed to implement another directive, on exposure to medical X rays.

Both radiation directives, agreed by the European Council of Ministers in 1984 (subject to ratification by national parliaments), are applications of the much older Euratom Treaty of 1957. This treaty is, in turn, one of the foundation stones of the European Communities and gives the European Commission wide powers to gather information and set standards of nuclear safety. The nuclear member states, in particular France, with nearly 70 per cent of its electricity demand met by nuclear power, are set firmly against such a role for the Commission. But in Brussels

it is felt that a clear, objective, international information system is urgently needed — otherwise the European public, including even the French, will lose confidence in nuclear power altogether. Last week Clinton Davies made a firm and specific proposal that such a system be set up, although he drew back from pressing for the wider powers that the Euratom Treaty would allow, but which for the moment seem politically inopportune.

As if to confirm the commissioner's views, the influential umbrella organization for European consumer associations, the Bureau Européen des Unions des Consommateurs (BEUC), which receives a small grant from the European Commission, also recommended such an international reporting system last week. In a 35-page report which collated official and published information on national responses to the Chernobyl disaster and its fallout, BEUC contrasted, among other things, the 3,700 becquerel per litre radiation level prescribed in France in milk with the 500 bq l⁻¹ upper limit in the Netherlands and Belgium, and the 20 bq l⁻¹ imposed during the Chernobyl affair in the German Länder of Hessen and Hamburg.

BEUC, with its large public backing through the consumer associations, also recommends in its report that the Commission adopt its full powers under the Euratom Treaty; that it set up a truly independent scientific committee to make recommendations on standards; that it prepare a European-wide contingency plan for any future accident, including one at a chemical plant; and that it develop a long-term post-Chernobyl research programme.

Robert Walgate

Soviet/West German cooperation agreed

Hamburg

WEST Germany and the Soviet Union have agreed on closer cooperation in science and technology. Foreign Minister Hans-Dietrich Genscher signed a framework treaty in Moscow two weeks ago which covers three sub-agreements on nuclear power, medical and agricultural research. Fifteen two-year programmes were agreed.

Then negotiations began in 1973, but soon came to a halt. Only when Chancellor Helmut Kohl met First Secretary Gorbachev in 1983 did both sides agree to continue the talks. A draft was ready in 1984 but in the new treaty the ministers add a note of their intention to work out a fourth,

additional sub-agreement on environmental protection.

The framework treaty is of particular importance in the light of Soviet efforts to make their nuclear power safe. The agreement includes clauses on exchange of information and scientists and the organization of meetings, exhibition and courses. Basic research may even be performed jointly in shared laboratories, but it is not yet clear what projects will be tackled. On the political side, the treaty is sure to help the progress of negotiations with East Germany on a similar treaty. Discussion began in 1973 but no agreement has been reached even after 27 meetings.

Jürgen Neffe

US-Japan trade

Truce agreed in chip war

Washington

Just minutes before midnight on 30 July, Japanese and US negotiators reached a comprehensive agreement on trade in semiconductors. Under the terms of the five-year agreement, the Japanese government will both help US semiconductor companies to increase their share of the Japanese market and stop Japanese companies from "dumping" chips on international markets. The US industry is well pleased with the agreement. But the industry knows that a key question remains; can it be enforced?

Reaching an agreement before midnight on 30 July was crucial. That was the statutory deadline for a Department of Commerce decision to impose permanent dumping duties on erasable programmable read-only memory chips (EPROMs). The Commerce Department had already made a preliminary judgement that Japanese manufacturers were dumping EPROMs — selling them at less than fair value considering their production costs — and chip importers have been posting bonds against possible imposition of duties. Under the agreement, both the dumping case against EPROMs, and a similar case against 256 kilobyte dynamic random access memory chips (256K DRAMS) with an 1 August deadline, will be suspended. In exchange, the Japanese government agrees to ensure its manufacturers sell products only at "fair market value". Also suspended is an unfair trading case being pursued by the office of the US Trade Representative, which could have resulted in sanctions against Japan.

To increase the US share of the Japanese semiconductor market, Japan will establish an organization to assist foreign manufacturers' sales efforts, and will also promote long-term relationships between US manufacturers and Japanese semiconductor users. While there is no specific target, the US market share in Japan is expected to increase to 20 per cent by the end of the five-year agreement. US sales at present account for 8–10 per cent of the Japanese market.

Another key element of the agreement, according to George Scalise of the Semiconductor Industry Association (SIA), is that US companies manufacturing in Japan will be given the same rights and privileges as Japanese companies, which is likely to increase the market share for the US industry.

In the week just before the signing of the agreement, Japanese manufacturers made a large number of sales to US customers. While the agreement on EPROM dumping became effective on 31 July, 256K DRAMS can be shipped to cus-

tomers at any price agreed before 1 August until 15 September. After that, fair market pricing will prevail. But US industry spokesmen concede that a large number of chips can be delivered to US customers during that time. Sales of 256K DRAMS now account for 17 per cent of the total US semiconductor market.

To prevent future dumping, Japanese companies will provide data on pricing of EPROMs and 256K DRAMS to the Department of Commerce on a regular basis. The Japanese Ministry of International Trade and Industry will keep track of pricing data on other semiconductors, as well as products to be shipped to markets other than the United States. The United States

can request immediate 14-day consultations to resolve dumping questions and retains the right to initiate its own anti-dumping investigations that could result in the imposition of dumping duties. The issue of third-market sales, and the possibility that chips would be dumped in small countries for trans-shipment to major customers at cut-rate prices, proved especially divisive in the negotiations. Although Japanese companies never admitted to dumping during the negotiations, Michael Gadbaw of SIA says they nevertheless "promised never to do it again".

What gives US negotiators confidence that Japan will adhere to the terms of the agreement is the threat that the suspended dumping and unfair trade cases can be reinstituted. But many believe that it will take a lot of work to turn these commitments into reality.

Joseph Palca

Technology journals

Time to learn Japanese

Washington

FEARFUL that US industry is not doing enough by itself to tap the voluminous output of Japanese scientific and technical literature, the US Senate is expected this week to agree to set aside \$1 million per year for efforts to make access easier. How the money will be spent has not yet been decided, but a possibility is that special representatives in Tokyo of the US Commerce Department will seek out the best from the Japanese literature and ensure that it is available in translation to US researchers.

Even supporters of the measure acknowledge, however, that the amount specified will do little to right the huge imbalance in the flow of technical information. The total number of US translators capable of handling difficult technical material in Japanese is small, perhaps only 200, with most of them working for one company. Japanese efforts to translate Western literature into Japanese, by contrast, are impressive: over 5,000 scientists and engineers are said to abstract routinely some 10,000 foreign and domestic journals, as well as other sources.

The Japanese Technical Literature Act directs the US Secretary of Commerce to redirect \$1 million from other Commerce Department activities to the monitoring and dissemination of Japanese technical developments. So far, the department has agreed to find only \$250,000. The act requires the department to consult businesses, professional societies and libraries in order to find out what is in most demand; it must, however, avoid offering material already sold by commercial companies.

A probable formula is that the Commerce Department's International Trade Administration will represent the Nation-

al Technical Information Service (NTIS, also part of Commerce) in Tokyo, and will then offer the information culled to US industry and academic institutions for a nominal fee. NTIS already monitors some Japanese government publications, but spent only \$18,000 last year on translation. The future of NTIS is, however, uncertain; the federal government has proposed turning over the agency in whole or in part to the private sector, and NTIS staff fear they may never see the money. The International Trade Administration, in contrast, already has ideas about what it would do with the promised \$250,000: produce another two of its in-depth reports on Japanese technology, four of which were produced recently for \$500,000 and were sell-outs.

By far the largest Japanese translating operation in the United States is the private-sector Japanese Technical Information Service (JTIS) of Media, Pennsylvania, a subsidiary of University Microfilms. JTIS (which claims to employ most of the qualified Japanese translators in the United States) abstracts some 600 Japanese journals for its industry clients and offers full translations as requested. But at \$5,500 per year for a subscription to its abstract journal, sales have been restricted to major companies, with only 150 or so paid-up subscribers.

Justin Bloom, a former science adviser at the US Embassy in Tokyo, says that if JTIS's service fails to prosper it will show there is "no demonstrable US interest" in access to Japanese literature. But he is sceptical about the new act, describing it as "laudable in principle but meaning little in practice". Bloom says that in Tokyo, even with a staff of 22, he was hardly able to make an impact in monitoring Japanese technical literature.

Tim Beardsley

European biotechnology

Parliament giving up alcohol

THE giant Italian food and agricultural company Ferruzzi is set to fight back after the European Parliament struck a blow against its plans to convert Europe's grain mountain into fuel alcohol. The plan, according to Ferruzzi, would create jobs, be environmentally benign, and give farmers a new deal. But, says a report adopted by parliament before the summer recess, the project would kill jobs in the oil industry and could produce far more pollution (in the form of biological oxygen demand) than all Europe's sewage. In Brazil, where a similar project has been under way since the 1970s, up to half a million jobs have been created, 80 per cent of them unskilled, says the report. There are no studies available, however, to indicate the possible impact in Europe, where educational and skill levels are higher and where technology is often cheaper than labour.

Ferruzzi, the company whose proposal led to the reports, was recently referred to the British Monopolies Commission for its thinly disguised attempt to take over British Sugar, the UK beet monopoly. But this has been only the latest event in the saga of the expansionist group, which already controls most of the sugar production in Italy and has a controlling stake in the leading French sugar manufacturer Beghin-Say. Control of British Sugar would give Ferruzzi one quarter of the sugar market of the whole European Economic Community (EEC). Through its alcohol project, the company has shown an almost equal interest in grain.

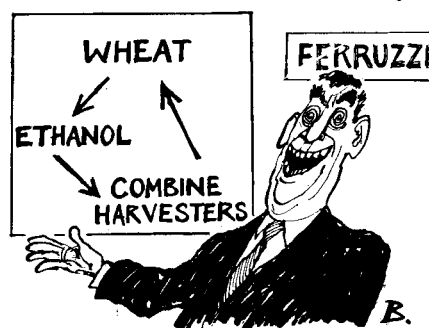
Ferruzzi's grain-to-alcohol plans would leave Scotch whisky producers standing. The group has proposed 12 conversion plants, costing upwards of \$560 million, and designed to take 15 million tonnes of cereals a year for conversion to alcohol.

The Ferruzzi plants would be built in France, where 94 per cent of Europe's grain surplus is grown (according to the European Parliament report). Naturally enough, the French farm minister François Guillaume, an ex-farming trade unionist, supports the Ferruzzi plan, and so do most of the French members of the European Parliament, but this was not enough last month to reject the parliamentary report, which itself rejected the production of bioethanol from raw materials "in present market conditions". The resistance to a large, precipitous bioethanol programme cut across all countries (other than France) and all parties; and although the parliament has little real power, its position will certainly give political weight to the European Commission (the EEC secretariat) which has also rejected the Ferruzzi proposal.

The principal reason for rejection is that the project is drastically uneconomic with-

out vast EEC subsidies. Ferruzzi's plans would require grain at world market prices. The present EEC subsidy to grain farmers, guaranteeing the sale of their grain at inflated European prices, would therefore have to continue. Moreover, this would still not bring the alcohol down to saleable prices as a fuel additive, where it could displace either petrol itself or the

In other words, perpetual motion!



new lead-free anti-knock agents. At present prices of oil and of anti-knock agents, governments would also have to offer tax reductions on any alcohol-containing petrol sold, the Ferruzzi group says.

Such arguments would seem to rule the whole proposal out of hand — and certainly the canny Dutch ex-economics minister, Fran Adriaenssen, who is now European Commissioner for Agriculture, rejects it — if it were not for the renowned strength of certain European agricultural lobbies, particularly the French. Ferruzzi's president, Dr Paul Gardini, has promised that he will not let the issue rest. The possible carcinogenicity of some proposed

anti-knock agents is one new line to try.

Meanwhile, therefore, the vote at the European Parliament is just one further move in the game, which is continuing through a \$200,000 study on the social and economic impacts of a European gasohol programme. This independent report should be ready in draft by November, when it could in principle receive attention by the Commission during the British presidency of the European Council, the Commission's decision-making body. But Britain, committed to reforming the over-productive and costly Common Agricultural Policy is thought to see European gasohol as yet another "black hole" of European subsidy, and will not aid the Ferruzzi project even if the study recommends it.

The European Parliament did not reject the development of bioethanol plants on a smaller scale, and at smaller rates of subsidy, than envisaged by Ferruzzi, however. Small plants could be of use to remote rural areas and parliament recommended Commission support for research and pilot projects along these lines. On the whole, however, the European Parliament, which has a higher representation of scientists than probably any national assembly in Europe, is of the opinion that the Ferruzzi plan would do damage to the more cautious and reasonable plans of the Commission to introduce biotechnology to European farms under its proposals for "stimulating agro-industrial development". These, involving pilot studies from post-research field trials of new crops to biotechnology-based processes, are not billed to remove the European food surpluses next year, but could, Commission officials hope, sow the seeds of a new European agriculture. A failed bioethanol programme could set such proposals back by years.

Robert Walgate

Transplant donor held up by red tape

WHEN Dr Robert Gale, the bone-marrow transplant specialist, flew to Moscow in May to treat the victims of the Chernobyl disaster, he took with him an essential piece of equipment for sorting cells, developed at Israel's Weizmann Institute. He also took an urgent request for help from an Israeli biologist, Michael Sherman, who himself needs a bone marrow transplant. Ironically, until he developed leukaemia last year, Sherman was employed at *Inter-yeda*, a commercial company established to develop innovations based on research conducted at the Weizmann Institute.

Michael Sherman is an emigrant from the Soviet Union, who settled in Israel some six years ago. The only possible related donor would be his sister, who still lives in Moscow. In February, as soon as she heard of her brother's illness, she immediately applied to OVIR, the Soviet

passport department, for permission to go to Israel to donate the necessary marrow. There was no immediate reply, but after Gale's visit, the sister and her husband and children were called to the OVIR office. They were informed that a temporary visit to Israel on a Soviet passport was impossible due to "bureaucratic difficulties", and were advised to apply for permanent emigration to Israel. On 2 July, therefore, they filed the appropriate papers.

Shortly afterwards, Sherman says, "unpleasant things" began to happen. His sister was demoted to a lower-paid job, and, now "they are examining the possibility of firing her". It now appears, he says, that OVIR is treating the case as a routine request to emigrate, with all the delays that involves. And that, in Sherman's case, could well be too late.

Vera Rich

Plant research

Success breeds privatization

THE British government is to go ahead with its plan to sell off its institutes for the development and marketing of new varieties of plants. The government has long seen the sale as politically desirable, given its philosophy that the role of the private sector should be expanded. But it was only last week that a merchant bank confirmed that the sale will also be financially profitable.

The two organizations to be sold, the National Seed Development Organization and the Plant Breeding Institute (PBI), might seem to form a natural couple. PBI, belonging to the Agricultural and Food Research Council (AFRC), runs large-scale programmes in plant breeding which have produced nearly all the new varieties sold by the National Seed Development Organization. This has been a profitable business, generating £4.65 million for the government last year. Given that support for plant breeding research at PBI cost just £2.5 million last year, the institutions are bound to seem an attractive buy.

In the short term, researchers in plant breeding may benefit from privatization. In recent years, they have suffered the same cuts in research council funds that have been felt elsewhere and one breeding programme, for *Triticale*, the family that includes grasses and cereals, came close to being abandoned. Private industry should provide funds more closely related to profitability. But there must be some

The new owners want you to concentrate on improving coca-leaf yields...



doubts about the longer term. Before privatization, PBI's molecular genetics and cytogenetics sections will be split off and combined with other AFRC institutes to form a new Plant Sciences Institute. That, according to PBI's director Professor Peter Day, will threaten a much admired quality of the institute, its ability to "couple disparate activities" under one roof. Whether it makes sense to remove the plant breeding part of the institute from its more basic molecular genetics research section just when the impact of molecular genetics is beginning to be felt must be a matter of controversy.

Much will depend on the buyer. The favourite is the Agricultural Genetics Company, a private company partly own-

ed by the British Technology Group that was set up in 1984 and given first option to develop plant biotechnology discoveries made at AFRC institutes. Although the company is well placed to ensure that fruitful cooperation between the privatized institutes and the research laboratories of AFRC continues, it lacks size. It is still a small organization with fewer than 30 staff, based at Cambridge's Science Park. Big multinational agricultural companies are bound to recognize the appeal of the two institutes and to be able to offer far more money for them. The only reservation they are likely to have is that the Agricultural Genetics Company has already gained the right to commercialize

many of PBI's discoveries. Whether the government will accept the Agricultural Genetics Company's view that it is "the natural vehicle for maintaining the integrity and future development" of the institutes within the private sector should be decided within the next few months.

The future of the remaining "plant sciences" fragment of PBI is unclear. The new Plant Science Institute will be completed by the addition of the John Innes Institute, the Unit of Nitrogen Fixation at the University of Sussex and a small part of the Rothamsted Experimental Station. But no provision has been made to bring the various parts together at one site: the "institute" will be more of an administrative convenience. Again, it is not clear if AFRC will itself profit from the sale of PBI or whether the proceeds will accrue directly to the Treasury. **Alun Anderson**

US-Soviet exchange

Relations thaw in the far north

Washington

AN agreement between the University of Alaska at Anchorage and the Siberian Branch of the Soviet Academy of Medicine to study problems of human adaptation to circumpolar living was endorsed by a Soviet delegation visiting Washington last week. But last minute uncertainty about official Soviet participation pointed up the fragility of US-Soviet relations.

For five years, Ted Mala, associate professor of health sciences at the University of Alaska, has been trying to work out an agreement with Siberian scientists to study health issues relating to life in the far north. Mala wrote to Soviet leader Mikhail Gorbachev, who responded favourably to his suggestions for cooperation, and arranged meetings with officials of the Soviet health ministry as well as the medical workers' union. The US-Soviet Exchange Initiative Office, established after last winter's summit meeting between President Reagan and Mr Gorbachev to encourage people-to-people exchanges, supported Mala's efforts.

Last week an agreement between the University of Alaska and the Soviet Ministry of Health was due to be signed through the US-Soviet Exchange Initiative Office, and Mala was summoned from Alaska for the signing ceremony. But on arrival in Washington, the Soviet delegation declined to sign a formal agreement, saying the exchange could take place only under health agreements in effect since 1972. The delegation did sign what amounted to a memorandum of understanding saying that Mala could visit the Soviet Union to complete arrangements for the exchanges, but formal plans would have to be approved next by the US-USSR Joint Health Committee formed under the 1972 agreement — a committee

that has not met for seven years.

Although health exchanges between the United States and Soviet Union have never stopped, there has been a slowing down in the past seven years. In 1972, the two countries signed two self-perpetuating agreements to cooperate in health sciences. The Joint Health Committee, consisting of senior health officials from both countries, met each year, but the United States cancelled the 1979 meeting following the Soviet invasion of Afghanistan. In recent years the climate has been changing, and a 1984 speech by President Reagan called specifically for the renewal of cooperation in the health sciences, a call given added impetus by last year's summit meeting.

A three-step plan has been worked out to improve relations. Surgeon General C. Everett Koop and Dr James Mason, director of the Centers for Disease Control, will visit the Soviet Union in October to discuss cooperation in the realm of public health. James Wyngaarden, director of the National Institutes of Health, will make a similar journey in November to discuss biomedical cooperation. Finally, the Joint Health Committee is to meet again next spring after nearly eight years.

As Mala's proposed exchange is strictly a private agreement between the University of Alaska and the Soviet Institute of Medicine, the US government has no control over what Mala plans to do. But the Soviet Union is placing things on a government-to-government basis. Harold Thompson, deputy director of the Office of International Health at the Department of Health and Human Services, says the US government is not happy with that arrangement, but does not feel that it should be allowed to interfere with exchanges. **Joseph Palca**

US space

Continuing problems all round

Washington

THESE are trying times for US space scientists. The space shuttle is grounded until at least 1988, and when flights resume, competition for cargo space will be fierce. While there is discussion of launching scientific missions on expendable launch vehicles (ELV), for the time being nothing is decided. Planetary scientists are among the hardest hit. At the end of last month, they learned that new weight restrictions mean that neither the Comet Rendezvous Asteroid Flyby (CRAF) mission nor the Saturn orbiter (Cassini) can fly aboard the shuttle. This news follows a decision to abandon the shuttle/Centaur upper stage that would have been used to take Galileo to Jupiter (see *Nature* 321, 800; 1986). Bruce Murray, professor of planetary science at California Institute of Technology (Cal-

That no major missions can be launched for at least three years does not necessarily have to be a drawback for planetary science, maintains Chahine. With a strong commitment from NASA, JPL would have no trouble assembling a team to plan new missions. Were planning to begin in 1988, a reasonable launch date would be the early 1990s, by which time either the shuttle or some other launch vehicle should be back in operation. Working on long-term projects is something JPL scientists are familiar with. What is needed, says Chahine, is a demonstration from NASA, in deed not just word, that planetary science will not be forgotten as NASA plans for the future.

JPL is not the only NASA centre to have taken a body blow from the delays in the space programme. Noel Hinners, director of the Goddard Space Flight Center in Greenbelt, Maryland, says Goddard also must cope with uncertainty. "Mentally, things are on hold", says Hinners. If the launch delays for Goddard projects such as the Gamma Ray Observatory or the Cosmic Background Explorer are of the order of one to two years, Hinners believes most key people will stay with the projects. But a delay of five years is a different story. Even keeping a launch team together for two or three years is tough, and Hinners reckons people will have to be laid off.

While the Hubble Space Telescope (HST) is in the same boat as the other major satellite launches, the delay seems to be less troubling to Garth Illingworth, deputy director of the Space Telescope Science Institute. He maintains that optical astronomers have never had a chance to become dependent on space-based observations, and can get by with existing ground facilities. "We are not really as subject to the feast or famine as the planetary people", he says.

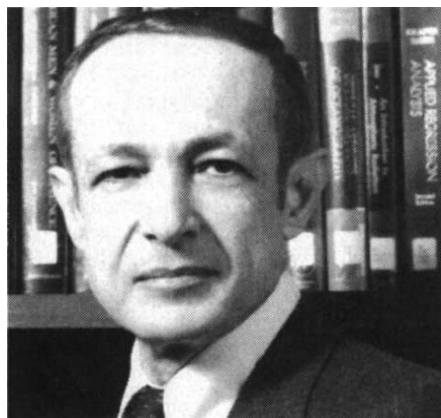
James Welch, Space Telescope project director for NASA, agrees that principal investigators are unlikely to leave the project. But keeping the engineering team intact will not be easy. Directing routine maintenance while the satellite is in storage is not a stepping stone to career advancement. One challenging engineering problem has, however, recently cropped up for HST. NASA's heightened awareness of safety issues has prompted a re-evaluation of the natural frequency of satellite subsystems. If they coincide with the frequency of shock waves generated by the shuttle, there could be serious problems. Determining these frequencies is not easy, says Welch.

Although NASA and the Reagan administration have not yet enunciated a clear policy on alternative launch vehicles

to the shuttle, most members of the space science community see a new fleet of expendable launch vehicles as crucial. Tom Donahue, chairman of the National Academy of Sciences Space Science Board, told Congress last month that he supports the use of the Air Force's new Titan 34D7 to launch deep space missions. Donahue favours moving to the Titan 34D7 to launch Galileo, Magellan and Ulysses (the joint mission with the European Space Agency to study the Sun's polar region) even if it means postponing acquisition of an orbiter or slowing down work on the space station.

If there is any bright side to the current upheaval, Murray believes it may be the study of Mars. The Mars Observer, the first of a series of low-cost planetary probes, is scheduled for a 1990 launch, and could still make use of the shuttle. Murray says the Soviet Union is planning two major missions to Mars in the next five years, and there have been non-governmental discussions about a joint United States/Soviet Union mission. These factors may make Mars programmes attractive candidates for new funds.

Joseph Palca



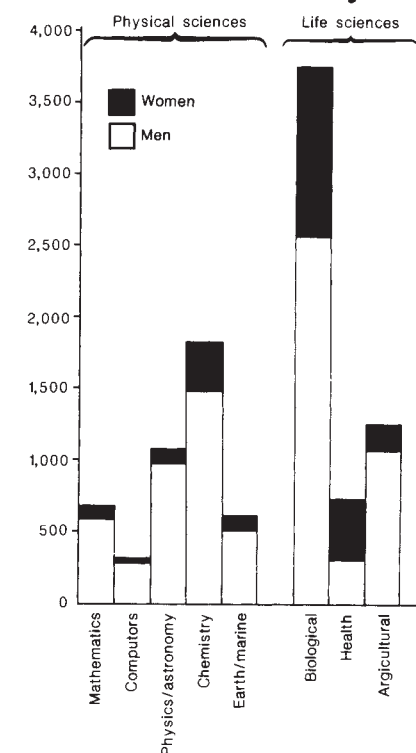
Moustafa T. Chahine — chief scientist at JPL.

tech), puts it bluntly, "the US planetary program has collapsed".

For the Jet Propulsion Laboratory (JPL) in Pasadena, the delays will be especially difficult. JPL is run by Caltech for the National Aeronautics and Space Administration (NASA). Although JPL is engaged in a variety of projects from remote sensing to astrophysics to defence-related research, Solar System exploration has been its bread and butter. Fully 20 per cent of JPL's 1985 budget was spent on three planetary probes, Galileo, Voyager and the Venus radar mapper Magellan.

The uncertain future is beginning to take its toll on the staff of JPL, says Moustafa Chahine, its chief scientist. Chahine says morale is still high from Voyager's Uranus flyby last January, but senior scientists now face tough decisions. The median staff age at JPL is 42, and scientists are at a crucial juncture in their careers. "Would you commit yourself to 10 years of uncertainty?" asks Chahine rhetorically. Although nobody has yet left JPL, Chahine is certain that some will do so in the coming months.

US doctorates by sex



THE numbers of doctorates awarded to men and women in the United States in 1985, taken from a report from the National Science Foundation division of science resource studies. In total 4,531 doctorates were awarded in physical sciences (3,817 to men, 714 to women) compared with 5,748 in the life sciences (3,893 men, 1,855 women).

New telescopes

Shining mirrors at Apache Point

Washington

USING a new mirror technology and a new design, a consortium of five universities is planning to build a 3.5-metre telescope in New Mexico's Sacramento Mountains. With a recently awarded grant of \$3.74 million from the National Science Foundation (NSF), the Apache Point Observatory is scheduled to begin operation in 1988.

Apache Point's mirror is a smaller version of the type of mirror likely to be used for the National New Technology Telescope (NNTT). It is cast using borosilicate glass and spun as it is cast, allowing a closer first approximation of the desired parabolic shape. The new techniques result in lighter and less expensive mirrors, bringing the cost of observatories the size of Apache Point to a level manage-

able by a small research consortium.

The five universities are not all equal partners in the project. The University of Chicago and the University of Washington will both contribute 31 per cent of the project costs, Princeton University and New Mexico State University 16 per cent and Washington State University the remaining 6 per cent. Bruce Margon of the University of Washington, chairman of the Astrophysical Research Consortium formed by the universities to build Apache Point, says viewing time will be allocated in accordance with contributions. Margon estimates the total cost of the project will be \$10 million.

Using private funds to build large observatories is a new trend. Several university groups have announced plans to build 7.5-metre telescopes, and the Keck Telescope, a 10-metre segmented mirror telescope that will be operated by California Institute of Technology and the University of California, is already under construction in Hawaii.

Several factors make Apache Point attractive. The observatory is the first designed for complete remote operation. Margon points out that this allows users to wait for appropriate conditions without having to make a pilgrimage to New Mexico. It will also be possible to divide a night's observation among astronomers at different institutions.

The performance of Apache Point may well exceed that of larger telescopes for certain problems. Keeping the structure housing the telescope to an absolute minimum will reduce atmospheric distortion caused by the heat from the observatory's own instruments. Sacramento Peak is also one of the darkest points in the United States, making it an attractive spot for observing extremely low light objects.

Perhaps most alluring to consortium members is that once built, Apache Point will be theirs to use as they please. Observing time is oversubscribed at the Kitt Peak National Observatory in Arizona and Cerro Tololo Inter-American Observatory in Chile. At Apache Point, says Margon, users will be able to tackle longer term and speculative projects.

Joseph Palca

More ocean dives

Tokyo

THE US submersible *Alvin* is scheduled to make its first visit to Japanese waters in 1987. Scientists from the United States and Japan have agreed plans for at least 85 dives to investigate the back- and fore-arc regions of the western Pacific.

Dives will be made in three areas; the Mariana Trough, a back-arc spreading centre, the Mariana fore-arc region which overlies the subducting Pacific Plate and the Sumisu back-arc rift northwest of the Ogasawara Islands.

Among the features to be investigated are a methane plume billowing up to 700 m above the floor of the Mariana Trough; diapiric seamounts bulging out of the seafloor of the Mariana fore-arc region; a chain of volcanoes that trends across the back-arc basin from the Northern Mariana Arc; and the Sumisu Rift which is thought to be a modern analogue of the setting for the Kuroko massive sulphide ore deposits on the Japanese mainland.

Funding for the project, which will run up a bill of a few million dollars, will be covered almost entirely by the US side.

Follow-up investigations of the survey area by the Japanese submersibles *Shinkai 2000*, and, when completed, *Shinkai 6500*, are expected to be made in the near future according to Hiroshi Hotta, director of the Deep-Sea Research Department of the Japan Marine Science and Technology Center (JAMSTEC), who will participate in the *Alvin* survey. In a recent review of policy, JAMSTEC decided to devote attention to the spreading ridges and rift basins in close proximity to Japan, such as the Okinawa Trough and Sumisu Rift. Also, in future, foreign scientists will be allowed to board *Shinkai 2000*. David Swinbanks

Timber trade

A little hope for tropical forests

At long last the world has an international forum that could help avert the total destruction of tropical forests. All the major tropical timber producing and consuming nations agreed in Geneva last week to put into operation the International Tropical Timber Agreement. Among its clauses are those aimed at "sustainable utilization and conservation of tropical forests and their genetic resources, and at maintaining the ecological balance . . .".

The agreement has been twenty years in the making and has been held up for more than a year by a squabble over the location of its headquarters. Only after a fresh meeting broke up in disarray was agreement finally reached: the headquarters are to be established in Yokohama and its executive director is to be Mr Haji Freezailah, deputy director-general of forestry for Malaysia. This arrangement neatly ties together the world's biggest tropical timber producer, Malaysia, and the biggest consumer, Japan. But argument is continuing over the budget for the new organization and nobody yet knows how effective a body it will be.

The UN-sponsored agreement does not establish a cartel and has no powers to fix prices or to regulate exports. But it does provide a talking shop for 41 nations that between them account for 95 per cent of the world's trade in tropical timber and, uniquely for a trade agreement, it has among its principal goals the preservation of tropical forests so that they may be

managed as a renewable resource. Other aims, also intended to benefit timber producers in the developing world, are to promote research and development in tropical forest management and utilization and to encourage the processing of timber in producing countries to promote their industrialization and export earnings.

The Scandinavian countries have taken the lead in introducing into the agreement clauses aimed at protecting tropical forests. Voting rights have been equally distributed among producer and consumer countries and if a majority can be found there is every chance that these clauses can be translated into action. The chief obstacle is likely to be lack of money. Several thousand million dollars need to be spent to halt tropical deforestation and the producer countries naturally feel that the bulk of the burden should be borne by the wealthy consumers. As a practical first step, the World Wildlife Fund is urging the appointment of an experienced forest ecologist to help run the agreement and prevent forest disasters; it also wants at least \$5 million to be set aside for conservation projects in the short term and says that prices for tropical timber should include the cost of reforestation, conservation and research on sustainable management. It is likely to be next spring, at the earliest, before the organization announces its initial budget and there is any indication of how successful it might be.

Alun Anderson

Academic oppression in Chile

SIR—On the afternoon of 1 July, we witnessed the siege and assault of the building of the School of Medicine of the University of Chile by a combined force of the military and the police. Military vehicles and troops first circled the quadrangle where the School of Medicine and its teaching hospital are located and then occupied both buildings. Among other actions, we saw a group of soldiers, armed with automatic weapons and with their faces painted black in combat camouflage, as they took twenty students as prisoners and made them lie face down on the floor of the hall of the School of Public Health.

Afterwards, another seventy students tried to take refuge in the classrooms of the department of physiology. The police broke in, took them out, and lined them up in the hall in order to arrest them. At that point, a professor implored the many faculty members gathered there to do something to prevent the students from being taken away. Her brave attitude created a vividly emotional atmosphere that allowed the dean of the faculty to persuade the police not to proceed to further arrests.

This episode was only one of the many assaults in the past weeks by combined military and police forces in several faculties of our university that have ended with violent arrests of students. These assaults typically include tear-gas bombing, the breaking into the university yards of heavily armed soldiers and vehicles and, in some cases, the destruction of installations and laboratory equipment. As a matter of fact, the action on 1 July was taken because the students were peacefully protesting against the arrest of 118 of their fellows, including the president and other members of the student council, which had occurred the day before inside the university main building. The student leaders were arrested invoking an article of the new Chilean constitution that allows special action to be taken against "terrorists that cause public alarm".

Student demonstrations have been aimed at ending the overt government intervention suffered by our universities since the 1973 military takeover; at the same time, the professors of the University of Chile have recently asked, through a referendum, for the resignation of the present principal of the university, a serving major general of the Army, and the election by the academic community of academic authorities.

On 2 July, Carmen Gloria Quintana, an 18-year-old student of the University of Santiago, and Rodrigo Rojas, a 20-year-old Chilean who had recently arrived from the United States where he was a resident, and who was collaborating as a computer programmer at the department

of physiology of the University of Chile School of Medicine, were found with burns that extended over 60 per cent of their body surface. This event has been well documented by the world press.

On 10 July, the government arrested some prominent leaders of social institutions, who had concurred in the creation of an Assembly of Civilians, asking for the return of the country to a democratic regime. Among those arrested were Dr Juan Luis Gonzalez and Dr Francisco Rivas, president and secretary of the Chilean College of Physicians, and Professor Patricio Basso, president of the Association of Professors of the University of Chile.

We think that these events should stimulate reflection and action by the international academic community. In experimental sciences, insights of widespread relevance are obtained not from the observation of phenomena in their usual context but rather from taking them to extreme conditions. In Chile, the relationship between university, society and the political regime has been brought today to a grotesque extreme.

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NORBEL GALANTI

CARLOS VALENZUELA

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AGS vindicated

SIR—In recent months it has been widely reported that scientists at Advanced Genetic Sciences, Inc. (AGS) "knowingly falsified" data in an application to the US Environmental Protection Agency (EPA). EPA has now "concluded that there is no information to indicate that (AGS) knowingly falsified its application".

The original allegations of data falsification surfaced during a congressional subcommittee hearing, and were based upon an unsigned two-page handout and an 8 × 10 inch photograph. These documents were supposed to demonstrate inadequate experimental design and plant pathogenic effects of the recombinant *Pseudomonads*, and were purportedly the subject of an AGS cover-up. EPA now accepts this "evidence" was inaccurate.

Unfortunately our vindication from the charge of falsification will not automatically reverse the damage that has been done to the reputations of individual scientists at AGS. The uncritical reporting of these allegations has caused much damage; scientific fraud should not be taken so lightly.

An article in *Nature* (320, 472; 1986)

referred to the allegations as though they were established fact, perhaps because of a misunderstanding of the operating procedure of EPA's Office of Enforcement and Compliance Monitoring. The process of developing an "administrative complaint" is interactive and EPA's initial charges were apparently made more in the spirit of prosecutor than in the role of judge. In fact, the charges of data falsification were not supported by EPA's own data audit, the basis for the final retraction of those charges, which had already been completed when the initial complaint was issued.

How and why did this situation lead to the publication of misleading, unjust and damaging reports? Somewhere between the deed and the reporting, the process of disseminating information went badly awry. It should be of concern to everyone when the news reaching the scientific community becomes subject to distortion by politics and sensationalism. It should be of concern to everyone that our case demonstrates the success of a frightening weapon—the gratuitous accusation of fraud.

J. BEDBROOK, P. DUNSMUIR,

J. LINDEMANN, T. SUSLOW, G. WARREN

Advanced Genetic Sciences, Inc.,

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Oakland, California 94608, USA

● We are glad to clarify that the allegation of falsified data appeared only the EPA's formal complaint, not in its announcement that AGS would be fined \$20,000 (later reduced). But should not EPA be more careful about giving currency to allegations such as these, especially when it knows them to be unfounded? — Editor, *Nature*. □

Parapsychology

SIR—Elitzur¹ criticizes Marks² for having omitted "representative" works. I know of no work that could be acceptable as a case for parapsychology. As for the extraordinary instrumental sophistication proudly presented, my question is why such a complicated mess is necessary for the basically rather simple claims of *psi* such as telepathy, telekinesis and so on. If they existed, their demonstration should actually be simple.

There is an explanation for the complexity. The more complicated the experiment, the more likely it is that it will have errors that will be attributed to *psi*. At the same time, it becomes easier deliberately to introduce errors and more difficult and time-consuming for sceptics to find them. If anything, the mentioned counter-hypothesis coincidence, artefact, self-deception and fraud become more likely.

AMARDEO SARMA

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1. Elitzur, A.C. *Nature* 321, 465 (1986).

2. Marks, D.F. *Nature* 320, 119–124 (1986).

New ways with quasi-crystals

While theorists continue to brood about the properties of quasi-crystals, experimentalists have begun to simulate them in the laboratory to tell how they behave.

WHETHER the unexpected fivefold geometrical symmetry of the manganese-aluminium alloy MnAl_{13} springs from an underlying quasi-crystalline structure, from twinning on a microscopic scale as Linus Pauling says (see *Nature* **317**, 512; 1985) or from a quite different phenomenon not yet identified, the whole idea of fivefold symmetry has enlivened several groups of people. That the theorists should have jumped in the new pool first is not at all surprising, which is why it is a little shocking that there is still no straightforward way of writing down a formula that will specify which points in three-dimensional space are the permitted vertices of a quasi-crystalline lattice. (The best approach is still to suppose that a three-dimensional quasi-crystal is the projection of a six-dimensional lattice on a three-dimensional surface.) But now the experimentalists have joined the fray, with entertaining consequences.

That fivefold symmetry is unattainable in an infinite regular crystal lattice has been known since the allowable symmetries of crystal lattices were first enumerated in the nineteenth century. The quasi-crystal explanation of the MnAl_{13} alloy, whose strange symmetry was first recognized by Schachtman, remains essentially that suggested more than eighteen months ago by D. Levine and P.J. Steinhardt (*Phys. Rev. Lett.* **53**, 2477; 1984). A quasi-crystal is not a regular lattice in which all vertices can be reached from all others by displacements represented by integral multiples of some basic set of vectors. Instead, the argument goes, the directions of the lattice bonds drawn between nearest neighbours in the crystal lattice take only a finite number of values, but the distances between successive vertices along a line, far from being equal, as in a regular crystal, may take one of two (or more) values which are, of necessity, incommensurate — their ratio is an irrational number. In two dimensions, the prototype of a quasi-crystal is the Penrose tiling of the plane, a kind of party trick by which a plane surface can be filled by rhombi of two distinct shapes yielding local fivefold symmetry. In this case, the crucial irrational number is the old Greek golden mean, half the square root of 5 plus 1.

How do experimentalists weigh into such a field? Here is a neat example, based on the familiar observation in superconductivity that the magnetic flux within a sufficiently small superconducting loop

will ideally be quantized in the sense of being an integral multiple of the elementary quantity $hc/2e$, where h is Planck's constant, c the velocity of light and e the charge of the electron. Only integral multiples of this magnetic flux are compatible with the condition that the superconducting loop carries no electric current. The origin of the condition is the requirement that the phase of the wave function describing electrons in the superconductor should be unambiguously defined.

Now a six-person group based mainly at the University of Pennsylvania but including Levine (*op. cit.*) has devised a clever experiment to show that magnetic fields can tell the difference between lattices that are regular and those that are merely quasi-crystalline (A. Behrooz, *et al.*, *Phys. Rev. Lett.* **57**, 368; 1986). What this group has done is to build a pair of two-dimensional arrays of aluminium wires merely 50 nm in diameter, one of which is a quasi-crystal of holes in one dimension, the other almost a Penrose tiling. The group has then measured the superconducting properties of this array in the presence of magnetic fields of different strength with the objective of telling how quanta of flux are distributed among the differently shaped holes of the lattice.

Others have previously set out to tell what happens when regular (not quasi-periodic) superconducting meshes are immersed in a magnetic field. If the total amount of flux, supposed uniform, across the mesh is an integral number of flux quanta, no electric current need flow in any of the wires so as to ensure that the phases of the electron wave functions are well defined. One of the simplest ways of recognizing that state of grace is to measure the temperature at which the superconductive transition takes place. If the temperature is identical with that of the superconducting phase transition in the absence of a magnetic field, the inference is that the flux through each and every hole in the mesh is an integral multiple of the flux quantum. Otherwise the transition temperature will be reduced. Behrooz *et al.* refer to earlier results showing that when the total flux through a regular mesh is less than enough to provide one flux quantum through every hole, the transition temperature is most nearly normal when the field is enough to endow each hole in the mesh with a rational (as distinct from irrational) fraction of a single flux quantum.

That is an obvious starting point for the experiments now described. The simplest trick is to make a mesh from 50 nm aluminium wires which are regularly spaced in one direction but whose spacing in the orthogonal direction is that of the Fibonacci sequence, whose spacings are related by the irrational golden mean. For practical purposes, there are holes of two different sizes whose areas are irrationally related to each other. The experimental result is striking, even startling. The temperature of the superconducting transition is most nearly normal when the average magnetic flux through each hole amounts to a number of flux quanta which is a power of the golden mean. The inference is that the most energetically favourable distribution of the magnetic flux is one on which as many holes as possible are threaded by integral numbers of flux units, which is what would be expected. It is nevertheless remarkable that it should in principle be possible to measure the irrational ratio of the dimensions of the mesh simply by measuring the transition temperature as a function of total flux through all the holes.

Similar results have been obtained with a network analogous to the Penrose tiling of the plane, but where the characteristic irrational ratio is the square root of 2. (Forgivably, the authors say that they were unable to make a true Penrose tiling with their microfabrication apparatus, which is nothing to be ashamed of; they managed 20,000 holes of two different shapes related by the square root of 2.) Again the most favourable threading of the total flux through the different holes is that determined by the irrational number. The principle is merely that the best configuration is that when the quantum condition is most often satisfied. What the practical applications will be is anybody's guess, but there could hardly be a more vivid proof of the quantization of magnetic flux.

As it happens, a calculation of a more general system of this kind, a randomly organized superconducting network, has been produced by two Argentinian physicists, J. Simonen and A. Lopez (*Phys. Rev. Lett.* **56**, 2649; 1986). Again, the conclusion is that integral numbers of flux quanta are energetically favoured. Meanwhile, experimentalists are busily studying other quasi-periodic structures. Obviously there is a whole new world to explore.

John Maddox

Astronomy

First X-ray-ionized nebula

from A.C. Fabian

THE hottest objects in a galaxy, the binary X-ray sources, should produce the most highly ionized nebulae. Despite extensive calculations of the ionization state of gas around X-ray sources (McCray, R. *et al. Astrophys. J. Lett.* **311**, L29; 1977), no such nebula had been found until M.W. Pakull and L.P. Angebault studied the Large Magellanic Cloud carefully, as they describe elsewhere in this issue (*Nature* **322**, 511; 1986).

Spiral and irregular galaxies are dotted with nebulae excited by hot stars. The H II regions are ionized by the ultraviolet radiation from massive main-sequence stars and glow mainly through recombination-line radiation. The even hotter cores of evolved stars that have recently ejected their outer envelopes, the planetary nebulae, ionize substantial amounts of helium as well. Visible radiation from planetary nebulae give an estimate of the ultraviolet radiation field, and thus surface temperature, of hot stars. In principle, this is a relatively simple deduction if it is assumed that all the photons capable of ionizing gases such as hydrogen do so, and that the resulting photon luminosity in some recombination line can be estimated from the observations.

The major problem in the search for nebulae around X-ray sources is that they tend not to be in dense gas environments, so the luminosity of the nebulae is very low. Massive main-sequence stars, by contrast, are young and embedded in dense star-forming clouds. Moreover, planetary nebulae create their own dense surroundings in the ejected stellar envelope.

The X-ray ionized nebula discovered by Pakull and Angebault, LMC X-1, was the first X-ray source found in the Large Magellanic Cloud and is securely identified by them with an O7 III-V star. The star appears to be surrounded by a nebula of radius 5 pc that emits visible lines of hydrogen and ionized helium as well as of doubly ionized oxygen. The He II emission is simply explained by the recombination of He III ionized by extreme ultraviolet photons of energy greater than 54 eV. These photons imply a much harder spectrum than any normal star and are consistent with the X-ray source. The centring of the emission on the O7 star identifies it, or rather its binary companion, as the X-ray source.

The extreme ultraviolet spectrum of LMC X-1 is of particular interest because the source is a black-hole candidate. Pakull and Angebault estimate that the spectrum is flat below the X-ray observed photon energies of ~1 keV down through

the otherwise invisible extreme ultraviolet to the ultraviolet. This could be the thermal black-body emission from an accretion disk. The spatial extent of the surrounding He III zone is ~10 light years and so presumably indicates that the source has maintained that spectrum and luminosity for at least the past 10 years.

LMC X-1 is an unusual black-hole candidate as it seems to be stuck in a 'high-state' (White, N.E. & Marshall, F.E. *Astrophys. J.* **281**, 349; 1984). The other black-hole candidates such as Cygnus X-1 switch between 'high'- and 'low'-states for about a month every year or so. (A high-state is characterized by a higher luminosity and steeper (softer) X-ray spectrum and a low state by the opposite.) An X-ray nebula around Cygnus X-1 should then appear as a set of rings around the source marking the past few high-

states. Unfortunately, the other black-hole candidates seem to be in low density regions and the He II nebula more or less undetectable at present. Indeed, it is curious that LMC X-1 is in a high-density region. The formation of the compact object in a binary X-ray source is expected to impart a substantial velocity to the system by momentum conservation. LMC X-1 should then be a long way away from the region of its birth. Perhaps it just happens to be passing through a dense region.

Pakull and Angebault remark that the LMC X-1 He III region is similar to the narrow-line region of a Seyfert galaxy. We can expect more investigations of the narrow-line region of LMC X-1 to explore this similarity as well as more searches around other black-hole candidates and X-ray sources. Such investigations have already revealed a high-excitation emission-line region around another LMC binary, LHG83. □

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Palaeoanthropology

Human phylogeny revised again

from Eric Delson

A newly recovered cranium of one of the early human species called robust australopithecines may well force palaeoanthropologists to reconsider the evolutionary relationships among all *Australopithecus* forms. On page 517 of this issue¹, Walker and colleagues describe two specimens (a partial lower jaw and a skull lacking most of the teeth and part of the skull roof) found in sediments on the west side of Lake Turkana, Kenya, dated about 2.5 million years (Myr) old. Many of those who have had the opportunity to study a replica of the skull feel that it is the most exciting fossil hominid found since 'Lucy' in 1974 (ref. 2).

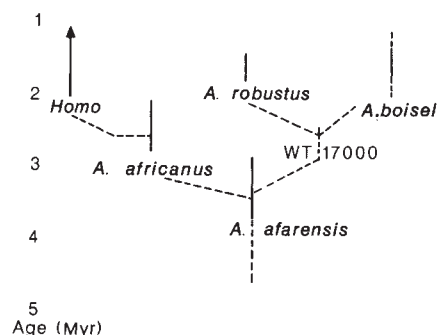
The record of early humans (upright bipeds with small canines and apparently already partly expanded brains in comparison to body size) begins about 4-5 Myr ago in eastern Africa. The first well-preserved remains are those from Laetoli (about 3.7 Myr) and Hadar (3.3-3.0 Myr) generally termed *A. afarensis*. This species is often thought to be close to the base of the radiation of Plio-Pleistocene humans, but opinions differ widely on details. Later australopithecine species include the 'gracile' *A. africanus* of South Africa, 3.0-2.3 Myr in age; 'robust' *A. robustus* (and perhaps a distinct *A. crassidens*) from 1.9-1.6-Myr-old caves in South Africa; and the hyper-robust *A. boisei* from eastern Africa, well known

between about 2 and 1.3 Myr ago. Based on his identification of isolated teeth and the revision of Omo chronology by Brown *et al.*³, Grine⁴ has argued that *A. boisei* may occur as early as 2.5 Myr ago in Lake Turkana basin. The earliest *Homo* fossils appear about 2 Myr ago in both eastern and southern Africa.

Although there is no consensus, most probably agree with the interpretation of Rak⁵ and of Kimbel and colleagues⁶ that *A. afarensis* is close to the common ancestor of two main lineages of early humans, one leading to *Homo* but unknown in the 2-3 Myr interval, the other passing through a 'gracile' stage to terminate in several robust species (of which the South African form is most like the common ancestor).

Others, especially Olson⁷, have suggested that *A. afarensis* is a mixture of two species, with most of the cranial and dental material being an early robust form based on shared features that are considered derived for the robust lineage, whereas Lucy and other less complete remains are like gracile forms and later *Homo*. Skelton *et al.*⁸ conclude that a third option is best: deriving both *Homo* and robust forms from *A. africanus*, with *A. afarensis* as an earlier ancestral stage.

The new robust fossils add to this already confusing picture. Walker *et al.*¹ make two main points in their analysis.



Possible evolutionary relationships among Plio-Pleistocene hominids. Solid lines, known ranges of taxa; dashed lines, probable range extensions and hypothesized relationships.

They argue first, that the new specimens can be included within the known species *A. boisei*; and second, that their antiquity now makes it impossible for South African *A. robustus* to have been ancestral to *A. boisei*.

In their Table 2, Walker *et al.* summarize the distribution among australopithecine taxa of 22 craniodental character states but their data do not, in my opinion, support allocation of KNM-WT 17000 to *A. boisei*. Of the 22 features, the new skull is apparently phylogenetically conservative in 11 and reasonably conservative in two more; in one (orbital height), it is similarly conservative in that only *A. boisei* is derived. In having the I^2 roots medial to the nasal margin, all post-*afarensis* forms are linked indeterminately. Thus, 15 of 22 features are phylogenetically neutral. In four characters, WT 17000 is grouped squarely with the other robust forms by derived features, but then only three remain to place the skull within the robust group. Of these, the shape of the orbital margins links it to *A. robustus*, whereas foramen magnum shape is shared with *A. boisei*; in neither character, however, is the state known in *A. afarensis* nor can an ancestral condition be readily determined.

The absence of a maxillary fossula appears to be a second link to *A. boisei*, but only if Rak's interpretation of development of the fossula in the *africanus-robustus* lineage and loss by *boisei* is accepted. If the fossula and related anterior pillars are functionally linked to canine root size, the fossula could even be independently derived in the two South African species — I consider this character as yet of indeterminate polarity. Walker *et al.* add to these tabulated features the supposedly *boisei*-like nature of the infraorbital, nasal and zygomatic root regions, but interpretations of those similarities differ among observers. We are thus left with only large overall size (and geography) as reasons for including WT 17000 within *A. boisei* and in the light of the quite distinctive mosaic of characters seen in WT 17000 (for example, prog-

nathic lower face and lack of a bare area where the muscle crests join), I would have assigned it to *A. boisei* only hesitantly at best. Further analysis (including study of Olson's³ characters of the mastoid and nasal region) could well show WT 17000 to be a rare case in which a new hominid merits description as a new species.

On the question of phylogeny, Walker *et al.* have argued that the great age of the new material falsifies the hypothesis that *A. robustus* or a similar taxon was ancestral to *A. boisei*. But it is possible (although undemonstrated) that a form resembling *A. robustus* inhabited southern (or eastern) Africa between 3 and 2.5 Myr ago, giving rise to all of the post-2.5 Myr robust species including WT 17000. As the new skull (and Lucy before it) showed, predicting fossil morphology in barren time periods is a risky pursuit.

But it is morphology and not time that reveals which taxa (or samples) are most closely related, and in this case, it seems best to interpret the new West Turkana robust forms as representing a population near the divergence of the southern and eastern variants. Although retaining

many character states seen in *A. afarensis* (and perhaps *A. africanus* as well), the form represented by WT 17000 presents several features characterizing all robust forms but few specific to either *boisei* or *robustus*. Its discovery leads paleoanthropologists to reassess the trends suggested by Rak for relating the gracile and robust species and the relationship of *A. afarensis* to all later species. The figure illustrates one such view. □

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Protein structure

Kinky variations of collagen

from John Galloway

DISCONTINUITIES compel our attention. They are a surprise, as much in the molecular biology of structural tissues as in everyday living. The principle of simplicity leads us to presume that a structure like the triple-helical collagen molecule, with its great uniformity of amino-acid sequence, will also be uniformly stiff and therefore straight or smoothly curving. Intuitively we do not expect it to kink abruptly. Perhaps that is why more than ten years have elapsed before a molecular model first aired in *Nature* in 1974 and elaborated two years later^{1,2} has now resurfaced³. In the intervening period the significance of the model has been considerably enhanced. Its interesting and novel feature — then only tentatively suggested^{1,2} but now formulated more concretely³ — namely that collagen molecules bend sharply at well-specified positions, now appears (or is thought to appear) in various tissues.

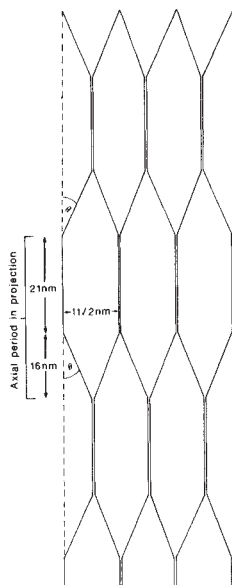
The collagen in question is that of the egg case of the dogfish *Scyliorhinus caniculus*. It differs from most known structural collagens in that it is secreted by epithelial cells rather than being produced by fibroblasts — a feature it shares, possibly significantly, with the better-known type IV collagen of basement membranes. It also differs from other, better-known, fibrillar collagens in forming a structure with an

axial period of 37 nm rather than the customary 67 nm. Under the electron microscope in transverse section it is seen to possess small crystalline regions in which a square lattice of side 11 nm is apparent. This is another unusual feature, as this sort of direct information has not been available for other collagens.

Briefly, the model (which is still schematic) is as suggested in the figure. The molecules (or groups of molecules) run parallel to the fibre axis along the edges of square prisms of side 11 nm and height 21 nm; they then bend more or less sharply through an angle of about 26° in a plane diagonally bisecting the prism; then run for another 16 nm; and then bend back and resume their course, again along the edges of square prisms but now displaced by exactly half the diagonal of the prism, that is, by $11/\sqrt{2}$ nm. This process continues for considerable distances. In this intriguing model, the simple relationship between successive prisms presumably results from segments of precisely the same length tilting alternatively 'right' and 'left'.

The lattice is, of course, extremely open. The side-to-side separation of the roughly close-packed collagen molecules in rat-tail tendon is about 1.5 nm, in marked contrast to the 11-nm separation seen in the egg-case collagen. Such an

open-work structure presumably provides an extended scaffolding or mesh which is then filled by the extra accessory proteins. It is difficult to avoid the conclusion that the large molecular tilt is the mechanism by which the open-work three-dimensional mesh is rendered possible at all, and kept mechanically stable. It is worth remembering that regular, plane-hexagonal meshes of collagen molecules have been proposed as a feature of basement membranes⁴. This may turn out to be a particularly useful comparison if, as is now speculated⁵, the egg-case colla-



Presumed arrangement of collagen molecules in planes diagonally bisecting the 11-nm square prisms, a molecular arrangement similar to the Z disks of striated muscle. $\theta = \tan^{-1} (11/16\sqrt{2}) \approx 26^\circ$.

gen is closely related to that of the basement membrane.

There remains the question of what features of amino-acid sequence cause the molecule to bend. Because little or nothing is known of the sequence of egg-case collagen, the question cannot be addressed directly, but it might be worth rehearsing some options borrowed from other, better-described collagens. Again because of the possible link, basement membrane collagen might offer a clue. Hoffmann *et al.* report⁵ that the flexibility of the type IV molecule varies in a marked way along its length and claim this to be consistent with what is known of the amino-acid sequence of the molecule, namely that it contains several interruptions in the otherwise regular sequence of triplets, Gly-X-Y. This approach has led to a view of the molecule as a series of stiff, triple-helical segments connected by short lengths of flexible non-helix.

A similar mechanism certainly seems to operate in the best-described collagen kink — that found in the molecule C1q of the complement system. All three chains, A, B and C, of the triple-helical parts of

the molecule have been fully characterized. Close to the kink in A, threonine is inserted between two triplets; an alanine substitutes for a triplet glycine in C and an entire extra triplet is apparently inserted into B (ref. 6).

On the face of it this interruptive mechanism would not be generally available to vertebrate fibrillar collagens where the amino-acid sequence within the triple-helical part of the molecule seems to be encoded exclusively by exons containing an integral multiple of 9m nucleotides ($m = 5, 6, 11, 12$ and 18) so that each exon encodes an integral number of triplets (unless the interrupting part itself always consists of triplets). Invertebrate collagens are less constrained. Could bending be brought about by mechanisms not involving residues actually interrupting the triplet sequence? For example, some regions of the molecule might simply be less thermodynamically stable. Hoffman *et al.*⁵ suggest that because imino residues are usually thought to confer stability, their absence over short regions might reduce local stiffness.

It is perhaps worth considering another possible mechanism in this class. The most striking single feature of the sequence of 1,014 residues in the triple-helical part of the $\alpha_1(I)$ chain is the presence of a solitary, idiosyncratically placed glycine. The sequence contains $1,014/3 = 338$ glycines, each in position 1 of the triplet, and one glycine, residue 311, in position 2. Such a small residue in place of a much bulkier one could well promote buckling, especially as the extra glycine is a feature of two out of the three chains (the $\alpha_2(I)$ chain does not have it).

At least two detailed models for the structure of rat-tail tendon invoke molecular kinking^{7,8} and both suggest possible kinking sites at the gap/overlap interfaces.

The 67-nm D-period, which includes one gap and one overlap region, is known to run through almost 234 residues and the whole triple helix runs through 4 gaps and 5 overlaps. Thus the overlap length is $1,014 - (4 \times 234) = 78$ (D/3) residues. The position of the interfaces are given by $234n$, where $n = 0, 1, 2, 3, 4$ and $234n + 78$. When $n = 1$ the interface falls at residue 312, that is, within one residue of the additional glycine.

Without a lot more work this observation, although interesting, is obviously no more than that. If every molecule kinks at every interface, what mechanism is operating at the other seven potential kinking sites? Perhaps the molecule does not kink at every potential site. At least one other case of a double glycine within a triple helix, residue 73 of the B chain of C1q, is not associated with kinking⁹.

If kinking is an important and universal feature of collagens then the range of ways in which it is contrived are worth studying, as are the ways in which genes conserve such crucial structural features. Reid⁹ has pointed out that in the gene encoding the B chain of C1q the single intron is located inside the codon for the glycine (other examples are known) within the inserted triplet and speculates that it might play a part in conserving the kink as a functionally important feature. □

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Muscle contraction

Through thick and thin

from Peter D. Chantler

Two recent papers report observations that both confirm and extend our ideas about how muscle contraction is regulated. Knox *et al.*¹ look at the role of tropomyosin in regulatory myosins, whereas Bennett and Bagshaw² investigate the non-specific divalent cation binding site of myosin, the major protein of the thick filament.

Regulation of muscle contraction can be divided broadly into two types: thin filament control and thick filament control. Thin filament control, typified by vertebrate skeletal muscle, is a function of actin, tropomyosin and troponin. During activation of muscle from the relaxed state, troponin senses the calcium signal,

causes tropomyosin to move in the groove of the actin helix and thereby removes the obstacle that prevented attachment of myosin heads (crossbridges) to actin — this is the well-known steric blocking model (Fig. 1a–c). In thick filament control, exemplified by scallop myosin, regulation of actin–myosin interactions is controlled by conformational events on the myosin head subsequent to calcium binding at a specific site (Fig. 2, site 2). Hence, there is no need for tropomyosin movement in this form of regulation: indeed, full regulatory behaviour is observed in the absence of tropomyosin.

A practical consequence of the steric blocking model, reported many years

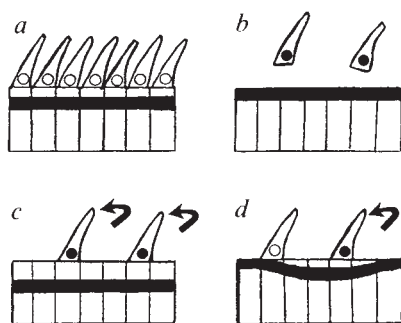


Fig. 1. The simplest explanation of rigor complex formation operative in thin filament regulation. Actin is represented as a set of 7 subunits; myosin heads can interact with only one subunit at once. Open circles, no bound ATP; closed circles, bound ATP; solid band, tropomyosin. Magnesium ($>10^{-3}$ M) is present in all cases. *a*, Rigor. No ATP; myosin heads bind to actin as a tight complex, pushing tropomyosin out of the way. *b*, Relaxed situation. ATP $>10^{-3}$ M, $\text{Ca}^{2+} < 10^{-7}$ M; myosin heads are prevented from binding to actin by tropomyosin assuming a blocking position. *c*, Active situation. ATP $>10^{-3}$ M, $\text{Ca}^{2+} > 10^{-6}$ M; myosin heads attach to, then detach from, actin in a cyclic, force-generating manner; tropomyosin is displaced to a non-blocking position. *d*, Rigor complex formation. Ratio of ATP to myosin heads < 1.0 , $\text{Ca}^{2+} < 10^{-7}$ M; rigor attachment of myosin heads, without bound ATP, pushes tropomyosin out of the way, thereby allowing heads possessing ATP to elicit force generation.

ago¹, was the ability of crossbridges to cycle in the absence of calcium and at low concentrations (less than 50 micromolar) of ATP. These observations were interpreted as follows: at low levels of ATP (no calcium, 1 millimolar magnesium) a finite population of nucleotide-free myosin heads exist. These form 'rigor' complexes with actin, in the process pushing tropomyosin away from its blocking position such that active myosin heads, possessing nucleotide, now have access to actin and can turn over (Fig. 1*d*). Similar results were not observed with scallop preparations³, a finding that has not been reported in full until now¹.

Because scallop adductor muscle is thought to be regulated solely by myosin-linked regulation, one would predict that there is no cycling of crossbridges in the absence of calcium at low levels of ATP, as such an effect seems to be a consequence of thin filament control. Nevertheless, several irksome observations have been made over the years: the X-ray diffraction patterns from contracting, rigor and relaxed molluscan muscle, interpreted as a movement of tropomyosin in the actin groove⁴; the presence of troponin subunits in some molluscan muscles⁵; and the apparent demonstration of a reversal of actomyosin relaxation at low ATP levels (by turbidimetric assay) in both scallop and squid muscle⁶. Knox *et al.*¹ principally address this latter point.

These authors examine the effect of low ATP concentrations on the relaxation of scallop myofibrils by ATPase, turbidimetric and light-microscope techniques. Using ATPase, they observe no increase in activity at low ATP concentrations in the absence of calcium, which leads directly to the conclusion that no crossbridge cycling is allowed in regulated scallop myofibrils at reduced ATP levels, exactly as predicted for a myosin-linked system.

Curiously, the turbidity measurements of Knox *et al.* confirm earlier observations⁶ that reported an increase in optical density at reduced ATP concentrations, both in the presence and absence of calcium. These results appear to point to the exact opposite conclusion — that cycling is allowed at low ATP levels. This apparent contradiction was resolved by light microscopy: comparison with myofibrillar shortening indicates that turbidity cannot be equated with contraction, but instead appears to be associated with shrinking and swelling of the myofibrils¹. Speculation that all forms of myosin-linked regulation will produce results similar to those obtained with scallop by Knox *et al.* should be tempered by the knowledge that tropomyosin can influence actin-activated ATPase rates in myosins controlled by light-chain phosphorylation.

Bennett and Bagshaw⁷, on the other hand, examine the rate of release of divalent cations from myosin subfragments (proteolytic portions of the molecule), in particular, from the high-affinity, non-specific sites known to be localized on the regulatory light chains of all myosins (Fig. 2, site 1). Several years ago, Bagshaw and Reed⁷ reported that the rate of dissociation of magnesium ions from this site on rabbit myosin subfragments was too slow to account for the rate of onset of contraction (complete within 100 milliseconds). They reached this conclusion by observing the decay of protein fluorescence consequent on chelation of the metal ion by EDTA in a stopped-flow apparatus. One criticism of this work is that measurement of the fluorescence signal was indirect and may have monitored a slower change on the protein that is subsequent to cation dissociation.

Bennett and Bagshaw⁶ have now repeated the measurements, this time monitoring events at the metal-binding site (Fig. 2, site 1), and have extended them to include scallop myosin subfragments. They have taken advantage of a neat spectroscopic trick to overcome the problem that Mg^{2+} is spectroscopically transparent under the conditions required to monitor its binding to protein. The trick is that manganese ions (Mn^{2+}) are not invisible by electron paramagnetic resonance and will give a tell-tale spectrum which becomes broadened when Mn^{2+} binds to the divalent cation site on the regulatory light chain, for which it com-

petes effectively with Mg^{2+} . Thus, by displacing bound Mg^{2+} , it was possible to monitor the decrease in signal of unbound Mn^{2+} , thereby obtaining Mg^{2+} dissociation rate constants from both rabbit and scallop subfragments that are similar to the earlier measurements obtained by protein fluorescence (0.05–0.06 per second)². Magnesium is therefore lost from these sites at a rate that is too slow for the ion to be replaced by activating concentrations of calcium (though one cannot exclude the possibility that some calcium is bound to the sites during a prolonged tetanus).

This result is no surprise to thick filament aficionados who have known for some time that these two high-affinity, non-specific sites exist in addition to the two calcium-specific sites (Fig. 2, site 2) located on scallop myosin, and it is this latter pair of sites that is responsible for the activation of contraction in scallop muscle. But for rabbit myosin there are no

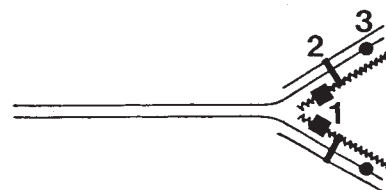


Fig. 2 Metal binding sites on myosin. Site 1 is the Ca/Mg site, located on the regulatory light chain; site 2 is the calcium-specific control site, found on scallop myosin (its exact location is unknown but it requires ligands from both the regulatory light chain and the myosin heavy chain); site 3 is the active site on the myosin heavy chain where ATP binds (usually in association with a divalent cation).

alternative sites: if one excludes the high-affinity regulatory light-chain sites as calcium switches, as the work of Bagshaw and colleagues appears to have done, then one must conclude that no on/off divalent cation switch exists on the thick filament of rabbit skeletal muscle, a result in harmony with many earlier observations. Although the activity of the ATPase site on rabbit myosin is undoubtedly influenced by the presence or absence of bound calcium and regulatory light chain, this influence is modulatory in nature, the true calcium switch being on the thin filament. The nature and location of the calcium-specific sites on scallop myosin is still unknown. □

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Fluid mechanics

G.I. Taylor and his influence

from Herbert E. Huppert

SIR Geoffrey Ingram Taylor OM, FRS was one of the giants of physical science in this century. His work was at the centre of research on the mechanics of fluids and solids and their application to aeronautics, chemical engineering, meteorology, mechanical engineering, metal physics and oceanography. He had a distinctive style of research that brought penetrating insight into the fundamental nature of almost all the problems he considered. At the same time, he was generally able to



design simple but effective laboratory experiments that tested, and usually confirmed, his theoretical concepts. Taylor was born on 7 March 1886 and, at a recent meeting* to celebrate the centenary of his birth, it was asked whether the style of his work is still alive, and of value, to contemporary research workers.

Taylor liked to describe himself as an amateur scientist — by which he meant that he enjoyed working independently, with a minimum of help from others, and, of central importance, for pleasure. His scientific curiosity led him frequently to initiate new areas of research, only to lose interest in them after he had discovered the fundamental phenomena of one field, by which time the possibility of developing another novel field struck his imagination. After the tragic sinking of the Titanic in 1912, Taylor was invited to be the meteorologist on the old sailing ship *Scotia*, which had been requisitioned to investigate the motion of icebergs in the North Atlantic. While on board, he grasped the opportunity to design instruments attached to balloons and kites flown to heights of 2 km from the masthead of the

ship. From these instruments, Taylor obtained the first reliable estimates of the transfer rates of momentum, heat and water vapour in the lower atmosphere. This work consolidated his interest in meteorology and turbulent diffusion and dispersion, to which he contributed so much in later years.

Taylor also originated important ideas in solid mechanics. A series of definitive experiments on various materials led him to develop a theory for 'dislocations' in metal crystals which predicted how cracks propagate and accounted for the observed strength of metal crystals. Motivated by conversations with Lord Rothschild, Taylor initiated the quantitative study of the swimming of microorganisms and, on the way, constructed a model of the tail of a spermatozoon from a metal-wire helix enclosed in a rubber sheath. The sheath rotated by the action of a wound-up rubber band anchored inside a head at one end of the helix. This model swam through glycerine at a rate that agreed with Taylor's theoretical prediction.

Because of the wide-ranging applicability of fluid and solid mechanics, and because of Taylor's ingenuity, his abilities were called on during the two world wars. In 1914 he helped to investigate the design and operation of military airplanes. While doing so he learnt to fly and make parachute jumps; operating as both experimenter and pilot he made the first pressure measurements over a wing in steady flight. During World War II he analysed and advised on a wide range of problems, including the rate of propagation of blast waves emanating from intense explosions.

Catalysis

Designing porous solids

from John M. Thomas

A JET of hydrogen impinging in air on platinumized, fibrous asbestos produces a spark. The famous nineteenth century German chemist, Döbereiner, capitalized on this phenomenon in the first commercial exploitation of heterogeneous catalysis. More than a million tinder boxes, containing compartments housing zinc and acid to generate the hydrogen, were made in the late 1820s and used as fire-lighting devices. The key to their operation is the degree of subdivision of the platinum engendered by the exceptionally large area of the fibrous support: no sparks are pro-

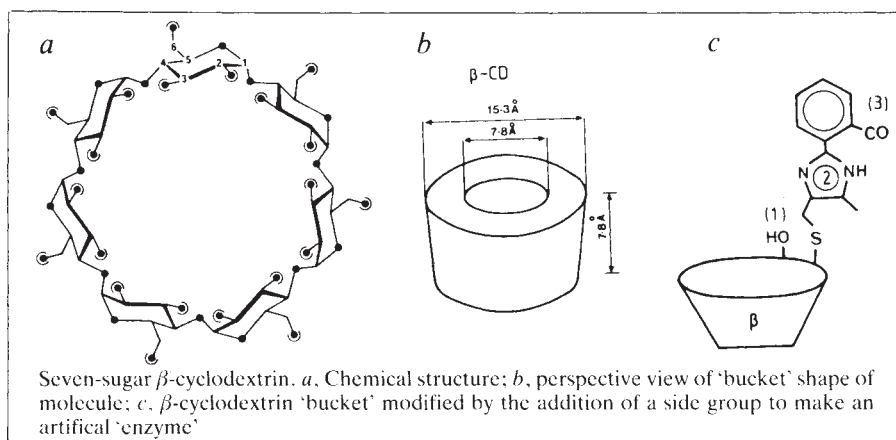
Taylor produced more than 200 scientific papers spanning the years from 1909 to 1972. An example of his inventiveness occurs in his design of an anchor. Unhappy with the holding power and weight of the traditional anchor, Taylor created a totally new design, with a single hook shaped like a double-bladed, symmetrical plough-share. This was much lighter than traditional anchors yet had the same holding power. These 'CQR' anchors are now widely used in small vessels and are still the best available.

Is it still possible, and useful, to carry out current research in the style of G.I. Taylor? He used his intuition and just sufficient mathematics and experimentation to develop extraordinary insight into the general nature of the area being considered. As was clear from the recent meeting, this style, although not easy to imitate, is currently being used to good effect in several areas of fluid mechanics. The conference confirmed that current research in fluid mechanics, in contrast to some other subjects, permits important contributions to be made on a very broad range of problems. These include dynamical problems such as chaotic and turbulent behaviour in the atmosphere and oceans and in industrial contexts. Another important area concerns the formation of 'finger' patterns when fluid of one viscosity displaces fluid of another within a confined space — a subject also started by Taylor, who wanted to understand how oil could be recovered from porous rock by driving it up ahead of a heavier, less viscous water layer pumped into the base of the rock. Such questions continue to demand all the imagination and ingenuity of Taylor's successors and will be solved by individual scientists or small groups working on their own — and for pleasure. □

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*Fluid Mechanics in the Spirit of G.I. Taylor, University of Cambridge, 24–28 March 1986.

duced with platinum wires, rods or foils of low area. Chemists ever since have been seeking new large-area solids, especially those that function as synergistic catalysts with a supported metal — such as rhodium for the control of automobile emissions, or palladium for the production of many pharmaceuticals. But industrial catalysis is not the only driving force in this quest. Porous solids are also required as plat-forms for a range of immobilized enzymes, as improved columns in adsorption and ion-exchange chromatography, and for effecting gas separations such as meth-



ane from carbon dioxide or nitrogen from oxygen.

In all this activity, several fundamental issues are raised, and some of these are touched on in the paper by Kijima and Matsui on page 533 of this issue¹. Achieving a large area is a necessary, but not always sufficient condition. As well as possessing pronounced adsorbability, the solid should be highly porous and exhibit shape — or size — selectivity. For certain applications it would be advantageous if it were capable also of chiral discrimination. The pore-size distribution must be so 'tuned' as to permit the ingress and egress of appropriate reactants and product molecules while excluding others. But why bother with the difficult task of tailoring solids of specific porosity when soluble, naturally occurring molecular sieves, such as the cyclodextrins, would appear to fit the bill?

Cyclodextrins, carbohydrates that on a molecular scale are like truncated cones or bottomless empty buckets (see figure), are the products of the enzymatic decomposition of starch. The α -, β - and γ -cyclodextrins have, respectively, six, seven and eight sugar units. The dimensions of the molecules confer upon their remarkable receptor — the interior of the bucket — properties for a range of guests. It is also relatively easy to add small side groups to them, thereby forming artificial or miniature enzymes (see figure). Bender and colleagues² have produced a miniature biological catalyst, rivalling the performance of chymotrypsin, by attaching the crucial functional groups of this enzyme to the rim of a β -cyclodextrin. And Armstrong *et al.*³, using β -cyclodextrin-bonded media, have recently succeeded in separating a wide range of enantiomeric drugs. These are important practical developments and are likely to initiate further effort in the design of specific receptors for molecular recognition and active sites for biological conversions.

By anchoring the cyclodextrins to benign inorganic solids of large area, it is much easier to separate product from reactant and to isolate one adsorbate from another. Zirconium phosphate, for

example, like the smectite clays⁴ and transition-metal chalcogenides⁵, is a robust, versatile layered solid which has attracted attention in view of its ability to intercalate organic molecules. Such layered solids may, by adroit chemical manipulations⁷⁻¹¹ be 'pillared' so as to convert them into highly microporous structures. Inorganic pillars, such as $\text{Fe}_3\text{S}_4(\text{P}(\text{C}_2\text{H}_5)_3)_3$ inserted into 1aS , yield stable microporous solids; whereas organic pillars, such as those based on the cyclodextrins, are less thermally stable. The latter class of designed microporous solids is likely to be useful for effecting highly selective chemical conversions and physical separations under ambient conditions.

It remains to be seen, however, whether, in designing porous solids to order, it is

better to pursue the strategy of inserting molecular or ionic fragments as pillars into available layered structures or to synthesize entirely new structures rich in wide-diameter channels and cavities. Rouxel *et al.*¹² have prepared a novel sulphide, $(\text{TaPS}_6)_8\text{S}_{10}$, in which tunnels about 9 Å long, circumscribed by eight Ta atoms, can reversibly accommodate ten-atom sulphur rings, S_{10} , and possibly other entities of comparable dimension. There are also reasons for believing that germanosilicates, or other so-called porotecto-silicates, based on truncated tetrahedra can, in principle, yield structures with cages of diameter 15 Å. □

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Molecular botany

Wonders of chloroplast DNA

from John Gray

THE complete nucleotide sequences of chloroplast DNA from a liverwort and from tobacco have been determined in two Japanese laboratories. On page 572 of this issue¹ Ohyama *et al.* report the arrangement of the 121,024 base pairs (bp) of the chloroplast genome of *Marchantia polymorpha*, and in a forthcoming paper in the *EMBO Journal*², Shinozaki *et al.* describe the 155,844-bp tobacco chloroplast genome. Despite the difference in size, the gene content and the organization of the genomes is remarkably similar, so similar in fact that the genes must have evolved from a common ancestor. This is of considerable interest for the evaluation of the evolutionary links between bryophytes and tracheophytes, which first appeared in the fossil record 350–400 million years ago. The completion of the nucleotide sequences reveals that the chloroplast genome has some surprises up its sleeve, both in the identity of its genes and their arrangement in the genomes.

Both the liverwort and tobacco chloro-

plast genomes conform to the normal arrangement for land plants in containing a large inverted repeat sequence separated by large and small single-copy regions³. The size of the inverted repeat sequence (10,058 bp in *Marchantia* and 25,339 bp in tobacco) largely accounts for the difference in size of the genomes. The single-copy regions are very similar in size in both plants. An inversion of approximately 30,000 bp in the large single-copy region is the major sequence rearrangement between the two plants. Inversions of this sort have frequently been found in chloroplast genomes⁴.

The genomes are predicted to contain more than 120 genes, including coding sequences for four different ribosomal RNAs; 30 or 31 transfer (t)RNAs; 55 proteins identified functionally or by sequence similarities with other organisms; and about 30 unidentified open reading frames (ORFs) ranging in size from 31 to 2,136 codons. There is a slight difference in the complement of tRNA genes in the two genomes; genes encoding

only 30 tRNAs have been identified in tobacco, but the liverwort contains in addition a gene encoding tRNA^{Arg} with the anti-codon CCG. In neither plant, however, are these tRNAs sufficient to translate all codons of the universal genetic code without invoking 'two-out-of-three' base codon recognition.

Before the completion of the two chloroplast sequences, all identified proteins encoded in the genome were involved either in photosynthesis or in the transcription and translation apparatus of the chloroplast⁴. The greatest surprise now that the sequence is complete is the presence in both species of ORFs showing considerable similarity to six genes encoding NADH dehydrogenase subunits in mitochondrial DNA. In tobacco, the four ORFs examined so far are all transcribed in chloroplasts. It therefore seems possible that these genes are functional in chloroplasts, and the identity of a functional protein complex containing the gene products will be of great interest. The possibility that these genes encode subunits of a chloroplast NAD(P)H-plastoquinone oxidoreductase is of great interest; chloroplast membranes of the green alga *Chlamydomonas reinhardtii* are known to contain an NADH-plastoquinone oxidoreductase^{5,6} that has a quinone-binding subunit of relative molecular mass 70,000 (ref. 7), approximately the same size as the *ndh5* (or *ndhF*) gene product.

Genes encoding components of ribulose biphosphate carboxylase, photosystems I and II, the cytochrome *b-f* complex and ATP synthase have all been identified previously⁴. But the liverwort sequence also indicates the presence of three ORFs encoding cysteine-rich 4Fe-4S ferredoxin-like proteins. Whether these putative proteins are part of photosynthetic protein complexes remains to be seen, but all identified proteins encoded by chloroplast DNA are parts of protein complexes that have at least one component encoded in nuclear DNA. This may be a regulatory feature, as first postulated⁸ by Ellis.

The completed sequences have added a few more genes encoding ribosomal proteins to the list, making a total of 19 ribosomal proteins in each chloroplast genome, although there are differences between the plants. The tobacco chloroplast genome contains the gene encoding ribosomal protein S16 which is not present in the liverwort genome, but the liverwort reciprocates by containing the gene encoding ribosomal protein L21 which is not present in tobacco. Slight variation in gene content between species is also indicated by the gene for elongation factor Tu, which is found in *Euglena* chloroplast DNA⁹ but not in either liverwort or tobacco genomes. The overall impression, however, is the great constancy in the

gene complement of different chloroplast genomes. The conservation of three genes encoding homologues of the α , β and β' subunits of *Escherichia coli* RNA polymerase is of special interest because the subunits of RNA polymerase had previously been reported to be nuclear-encoded¹⁰.

The organization of chloroplast genes is essentially prokaryotic. In several instances there is clustering and co-transcription of functionally related genes, with the gene order similar to that in *E. coli*. This is strikingly illustrated with the genes encoding ribosomal proteins; the cluster *L23-L2-S19-S3-L16-L14-S8-infA-secX-S11-rpoA* is identical to the gene order in the *S10-spc-a* operons of *E. coli*. Another prokaryotic feature is that of overlapping genes, and there are several examples in the chloroplast genome including *atp B/E*, *psb D/C*, *rsp3/rpl22* and *ndhC/p-sbG*. The last is the only example where two gene products are not part of the same protein complex, and this may perhaps indicate an error of identification or a previously unrecognized mechanism for coordinated regulation of two protein complexes.

Although essentially prokaryotic in organization and expression many genes appear to contain introns of various types. Six tRNA genes contain introns in the anticodon loop or, in the case of the gene encoding tRNA^{Gly}, in the D stem. An ORF in the intron of the gene encoding tRNA^{Leu} is similar to the ORF in intron 2 of the yeast mitochondrial gene encoding cytochrome oxidase subunit I. A further nine identified or putative protein-coding genes are predicted to contain introns, the most spectacular being in the gene encoding ribosomal protein S12, where the exons are located far apart on different DNA strands. If this constitutes a

functional gene, then a *trans*-splicing mechanism must operate. The mechanisms of splicing in chloroplasts promise to be an interesting subject for study.

The completion of nucleotide sequences of genomes of this enormous size invariably invokes the question: what is there left to do? The answer is plenty! The sequence constitutes the ground work for future studies on the expression and replication of the chloroplast genome. The ORFs require identification: what is the identity of the huge ORF 2,136 in the liverwort genome or the homologous ORF 1,708 in the tobacco chloroplast genome? The expression of the genes requires study, not just in leaves, but in all the other parts of the plant. Plastids are found in all plant cells and all contain identical genomes; the chloroplast is merely a plastid specialized for photosynthesis. Another question that the sequences have left unanswered is the identity of the origin of replication. Although *ars* sequences capable of replication in yeast have been identified, they do not map in the region of the chloroplast origin of replication in higher plants¹¹. Much has yet to be learned of the intricacies of chloroplast DNA. □

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Tumour immunology

MHC antigens and malignancy

from Hilliard Festenstein and Federico Garrido

EXPERIMENTAL evidence indicating that major histocompatibility antigens have an important role in host defences against the development and spread of tumours is rapidly accumulating. Various spontaneous as well as experimentally induced animal tumour cells exhibit quantitative and qualitative differences in the expression of both class I and class II antigens encoded by the major histocompatibility complex (MHC). Although the selective absence of MHC class I molecules is associated with enhanced tumour growth and metastatic capacity in some experimental models, in others apparently contradictory results have been obtained but little is known about the mechanisms involved.

We know even less about changes in expression of MHC antigens by human tumours. The progress that has been made in this field to date was the subject of a meeting* held last autumn.

The concept that the selective absence of H-2 class I antigens in mice favours the development of some malignant neoplastic tumours was supported by studies from three groups on the K36 AKR leukaemia (H.F.), on the T10 bladder sarcoma (G. Hämmerling; Heidelberg) and on the methyl cholanthrene-induced GR9 tumours in BALB/c mice (F.G.). Cellular

*Major Histocompatibility Antigens on Tumours, 2-5 October 1985, Granada, Spain.

clones derived from these tumours which either lack or have a relative deficiency of H-2K antigens tend to grow and metastasize more readily than similar cells transfected with and expressing an *H-2K* gene but that were originally H-2K deficient. Evidence was presented to suggest that surveillance by T lymphocytes is the most likely relevant anti-tumour host response in these experiments in that T cells recognize tumour-associated antigens only when associated with certain class I antigens (MHC restriction).

An apparently contradictory result is found (K. Kärre, Stockholm) in YAC -I tumour cells induced by Moloney virus where increased amounts of H-2 antigen expression is directly related to increased metastatic potential. But the host response in this case is apparently related to natural killer cell activity, which operates best in the absence of H-2 antigens on the target cells.

T-cell surveillance may be relevant however, as when large doses of these tumour cells are injected into preimmunized animals their growth is favoured by the absence of H-2 antigens. In individual primary AKR lymphomas the quantitative expression of H-2K and H-2D antigens correlates with the quantity of a murine leukaemia virus-encoded glycoprotein on the cell surface (P. Demant, Amsterdam). This could ensure the preferential elimination by cytotoxic T lymphocytes of cells with high levels of expression of viral antigens.

Although T-cell surveillance is clearly dependent on the presence of the relevant class I MHC molecule in most instances, there are exceptions in some AKR leukaemias (W. Schmidt, Essen) and in some human Burkitt lymphomas (E. Klein, Stockholm) which are resistant to T-cell cytotoxicity even though the relevant restriction elements are apparently present. These findings stress the importance of the heterogeneity of the quality and quantity of the host's immune responses to tumours.

As the level of expression of H-2 antigens on syngeneic tumour cells is relevant for host-tumour interactions, it is important to elucidate the mechanisms of control. Three presentations were in agreement in proposing that DNA methylation is likely to be as important in regulating expression of both class I and II MHC antigens and also the *Thy 1* antigen. C. David (Rochester) showed that Class II H-2 antigens are expressed after treating the BW 5147 thymoma line, with the demethylating agent 5-azacytidine; E.

Pareja (Granada) showed a similar result for class I antigens after treating an H-2-negative GR9 tumour cell clone with 5-azacytidine. He also showed differing methylation patterns that correspond to the different levels of expression of H-2 antigens on the various tumour cell clones.

F. Grosveld (London) came closer to identifying a site of action by showing that the methylation of the 5' flanking region of the *Thy 1* gene is responsible for the control of expression of this antigen. Possible transcriptional and post-transcriptional mechanisms mediated by transforming proteins of DNA tumour viruses that may also account for the regulation of class I antigen expression in SV40-transformed cells were indicated by P. Rigby (London). Ultimately these studies will open the way for the manipulation of host-tumour interactions through the regulation of syngeneic H-2 antigen expression.

Expression of aberrant H-2-like allo-antigens by tumour cells was originally described about 10 years ago by the group of one of us (H.F.), soon followed by similar observations of others. There is new molecular evidence for the expression of an H-2D^d-like specificity on an H-2K AKR leukaemia (H.F.) and Schmidt described an aberrant H-2K^b-like molecule which is responsible for rejection of a tumour induced by the Rous sarcoma virus that does not express normal syngeneic Class I antigens.

H. Schreiber (Chicago), M. MacMillan (Los Angeles) and R. Goodenow (Berkeley) discussed the well-characterized novel class I MHC molecules identified on the ultraviolet radiation-induced fibrosarcoma 1591. What is of interest here is that the genes encoding two of these molecules have a high degree of homology with H-2L which is not normally expressed on cells of the H-2^d haplotype.

Thus chemicals or viruses may induce gene rearrangements that cause derepression of silent genes, leading to the appearance of aberrant H-2-like allogeneic molecules. The multiple *Qa*- and *Tla*-like genes that have a considerable degree of homology with *H-2* genes could provide the genetic basis of aberrant MHC antigen expression. Such H-2-like molecules capable of eliciting an immune response may function as tumour-associated transplantation antigens, or be intrinsically suppressive in another genetic context. P. Demant (Amsterdam) and P. Stern (Liverpool) both showed class I-like products on embryonal carcinoma cells but disagreed on whether they were normal or aberrant products.

Expression of MHC antigens on human tumour cells was also considered. 'Extra' HLA class I-like specificities of the AW19 cross-reactive group on HTLV-I infected cells were described by D. Mann (NIH)

and were attributed to the high degree of cross-reactivity between virally induced tumour-associated antigens and these HLA specificities. P. Schrier and D. Ruiter (Leiden) and F. Ruiz-Cabello (Granada) both found a lower level of expression of class I HLA antigens in metastatic compared with primary melanomas but G. Parmiani (Milan) found no difference between the level of class I MHC antigens in metastatic and primary melanomas.

Difficulties are encountered in interpreting the results of some of the other solid tissue human tumours studied, where extreme heterogeneity of MHC antigen expression in individual cells within the tumours is found. This is true for several solid tissue tumours, including breast carcinomas (J. Peña, Córdoba and M. Perez, Granada) and gastrointestinal neoplasia (M. Moore, Manchester). In a study of colorectal carcinomas Hämmerling and Peña find a correlation between the loss of MHC class I antigen expression and the degree of cell dedifferentiation within the tumours. We believe that both these solid tumours and chemically induced tumours in mice arise from multiple clones having differing levels of expression of MHC antigens. Class II MHC antigen levels could thus be indicators for metastasizing melanomas (Ruiter; Parmiani).

C. Navarrete (London) reported differential expression and function of MHC class II antigens (DR, DQ) on various acute leukaemic cells and cell lines. These findings may have important biological consequences in host-tumour interactions as was implied further by the data presented by A. van Leeuwen (Leiden) on the expression of TCA- (Tla-like) antigens on several human leukaemias.

Another question concerns the association of MHC antigens with particular tumours. M. Zijlstra (Amsterdam) demonstrated in mice that alleles of class II *I-A* control the type of lymphoma that develops (T.B or non T. non B) with MCF 1233 virus. T. Oliver (London) showed a significant positive association between HLA-DR5 antigen frequency and the development of seminomas in man.

It appears the genetic basis for the expression of aberrant MHC antigen specificities on tumour cells is probably the result of a series of gene-conversion events and DNA rearrangements involving pseudogenes and genes encoding differentiation antigens and by various initiating factors including toxic chemicals, radiation effects and viruses and influenced by host lymphokines. □

Erratum

In the article by K.A. Browning *et al.* on atmospheric fronts (*Nature* 322, 114; 1986), Fig. 2 should have been rotated clockwise by 90 degrees to relate to the model simulation shown in Fig. 1.

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The origin of Japanese HTLV-I

SIR—Gallo and his colleagues¹ seem still to believe that African blacks came to Japan with the Portuguese in the sixteenth century, that HTLV-I (human T-lymphotropic virus type 1) adult T-cell leukaemia virus was transmitted from them to the Japanese people and then became highly endemic in Japan^{2,3}. But our studies^{4,5} strongly suggest that HTLV-I was already present in aboriginal Japanese in pre-historic times.

There are at least three ethnically distinguishable populations in Japan: the Ainu in the north the Ryukyans in the south and the Wajin, or "Japanese", inhabiting the rest of the country. In our examination of the prevalence of HTLV-I in these ethnic populations^{4,6}, we found that the incidence of virus carriers was highest in the Ainu and the Ryukyans, not the Wajin, and that the seroprevalent frequencies in adults over 40 years old of these three ethnic groups were 45.2%, 33.9% and 1.1% respectively.⁴

The Wajin, who constitute most of the present population of Japan, are considered to be descendants mainly of post-neolithic immigrants from the mainland in the Yayoi and the Kofun eras (300 BC to AD 600). The Ainu and Ryukyans, who share several common physical and genetic traits, are considered to be relatively pure descendants of native Japanese populations. The Ainu in particular are regarded as descendants of the native population inhabiting mainly northern Japan from the pre-agricultural Jomon period, more than 2,300 years ago.

The highly endemic areas of the retrovirus in Japan have been demonstrated to be restricted to (1) the most northerly and southerly regions and (2) isolated areas in other regions^{7,8}. These native populations have been relatively unaffected by the Wajin who are not HTLV-I carriers. In most other areas, however, the Wajin became predominant because of their higher technological skills, planting rice and making iron tools and arms, for example.

Gallo and his colleagues¹ state that "the Japanese word for monkey *amakawa* is... derived from the Portuguese word *macaco*, also meaning monkey, however, we do not call monkeys *amakawa*, but *saru*. Furthermore the Japanese word *amakawa* is derived from *Macao*, the Portuguese territory in China. It may be true that the Portuguese brought Africans and African monkeys to Japan, but there is no relation between the places where the Portuguese landed in Japan and the endemic area of HTLV-I^{9,10}. Among the Ainu people living in Hokkaido (the most northerly island of Japan) there are no monkeys living outside zoos. HTLV-I is prevalent, but Roman Catholicism, which

Gallo *et al.* say was imported from Portugal, is not. Thus, the hypothesis that Japanese HTLV-I derives from Africans brought by the Portuguese in the sixteenth century is inadequate. Alternatively, from our recent finding that HTLV-I is prevalent in northern Japan and the report of Gallo's group of the presence of HTLV-I carriers in the Arctic¹¹, we propose that, like simian HTLV-like virus, which is now found in Old World monkeys¹²⁻¹⁴, HTLV-I was originally prevalent in all humans, but that during human evolution it was lost from all but a few populations.

Finally, we think that it is premature to draw a conclusion on the origins of HTLV-I, because many populations have not yet been examined.

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Phosphoinositol more than skin deep

SIR—Touqui *et al.* may have been too cautious in the discussion of their paper which showed that the phosphoinositol (PI) cycle is responsible for activation of phospholipase A₂ (PLA₂) and initiation of the arachidonic acid cascade in platelets¹. Our current studies of epithelial growth control provide data that are in line with their hypothesis, allowing us to interpret the findings of the Paris group in a broader context. It has long been recognized that a tissue responds to injury in two ways — inflammation and regeneration — and that these are tightly coupled; indeed, in normal circumstances they are inseparable. From a teleological point of view this of course makes sense, but in mechanistic terms still awaits an explanation. We are now in a position to provide one, namely that both arms of the response are triggered by activation of the PI cycle.

As in certain other tissues, PLA₂ activity in human epidermis is calcium-dependent and appears to be inhibited by a lipocortin-like molecule²; the epidermal inhibitor also seems to be modulated by

phosphorylation³. Thus the findings of Touqui *et al.* may be extended to epidermis, a conclusion strengthened by the report that phorbol myristic acetate increases the synthesis of arachidonic acid metabolites by cultured keratinocytes⁴. These metabolites include prostaglandins (responsible for vasodilation) and chemotactic agents, such as leukotriene B₄, which initiate invasion of the tissue by granulocytes.

The link with proliferation was originally suggested by observations that protein kinase C, following activation by diacylglycerol, phosphorylates (and hence activates) a membrane antiport which exchanges intracellular H⁺ for extracellular Na⁺. In several cell types the resulting increase in cytosolic pH causes entry of resting (G₀) cells into the mitotic cycle^{5,6}. Again, extrapolation to epithelia seems reasonable, since phorbol esters applied topically to skin cause a dramatic hyperproliferation.

Further evidence comes from our unpublished observation that inhibitors of the antiport and/or protein kinase C, such as amiloride, abolish the recruitment of G₀ keratinocytes in human epidermis following experimental injury.

Thus the findings of Touqui *et al.* may well lead to a clearer understanding of the way in which a tissue responds to injury, one of the central problems in pathology. We would also note its relevance to psoriasis, a common skin disease with polygenic inheritance. The lesions of this disease are characterized by a continuous, chronic inflammation and secrete high levels of arachidonic acid metabolites⁷. The epidermis within the lesions is grossly hyperproliferative with a complete absence of G₀ cells⁸ and PLA₂ activity increases in the entire epidermis of the patient which seems to result from an over-phosphorylation of the inhibitor³. It would seem that the PI cycle may be a rewarding field for research into the pathogenesis of this disease.

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Faith in the physicists

Joseph Silk

In Search of the Big Bang: Quantum Physics and Cosmology.

By John Gribbin. Bantam/Heinemann: 1986. Pp. 413. Pbk \$9.95, Hbk £14.95.

SUSY GUTS is not a radical feminist, but a theory that is designed to unify the fundamental forces of nature. The reader will find this, along with many other esoteric facts, relieved by occasional gossip anecdotes, in the latest book from prolific science writer John Gribbin.

In Search of the Big Bang completes a trilogy of major works on what Gribbin considers to be the most important scientific achievements of the twentieth century. Previous volumes were devoted to the nature of reality and quantum physics, and to the origin of life: here we move onto the ultimate issue of the creation of the Universe itself. The faint of heart may dispute the status of our cosmic origins as an equal partner to these other matters, but Gribbin unabashedly takes us on a grand tour of modern cosmology.

The difficulty is that cosmology is a science starved of data and not readily amenable to controlled experiments. We must take each glimpse of the Universe as it comes, no matter how confused or obscure it happens to be. Remote galaxies appear as they were aeons ago, and thereby profoundly alter our normal sense of perspective if, for example, young galaxies were more luminous than the mature, nearby galaxies. It is as though we view the Universe through a distorting lens, and we have little sense of the nature of the distortion. And this applies to the galaxies, whose formation was only the most recent episode in the history of the Big Bang. Extrapolating backwards in time from the furiously receding galaxies we see today, we infer that the Universe was once a far more exotic, denser, hotter hostile place.

In many respects, the Big Bang is to modern cosmology what mythology was

to the ancients. To believe that we understand the very early Universe, the first microseconds of cosmic time, requires immense faith in the physicist's search for the ultimate union of the fundamental forces of nature, because direct evidence is completely lacking. Yet to the physicist, the vast energies attained in the immensely compressed primeval fireball that was once the Universe offer a unique testing ground for the latest theories of elementary particles. Exotic names, like photinos, strings or even superstrings, are reeled off as though they were the most natural state of matter, which perhaps they once were. Provided we accept theories of matter and gravity that are certainly correct at our present epoch and in our environment, then we are inevitably led by the Big Bang to a singular state near the origin of time.

Of course we are still awaiting the ultimate theory of everything, currently thought to be superstrings, which will explain the most important missing link in the puzzle, namely how it all began. Many physicists around the world are pursuing this ultimate goal that represents the union of gravity and quantum mechanics. However, we are not there yet, and opinions differ widely as to how far away we may be: according to Stephen Hawking, the end of physics is in sight, yet Sheldon Glashow argues that superstrings are only a mirage.

My only quibble with Gribbin's book is that he presents physicists and astronomers as gods whereas, in reality, they are no less fallible than other mortals. The Big Bang theory is an excellent description of the here and now, and of the not-so-long-ago and not-so-far-away. But one ought to take the extrapolations back to the begin-

ning of time with a healthy dose of scepticism. The Big Bang cosmology may yet be superseded, just as Newton's theory of gravitation was incorporated by Einstein into a new theory. Our more extreme extrapolations of space and time could prove to be equally false, or at least, incomplete.

In Search of the Big Bang is a remarkably readable guide, however, to the mysteries of cosmic creation. It is strong on personalities, from anecdotes about missed opportunities and Nobel prizes, to the story of the wealthy telescope builder and the mule skinner who paved the way to the realm of the nebulae. The first third of the book represents a historical development of cosmological data, and the second third provides a readable description of the classical Big Bang theory. The remainder is devoted to quantum physics and the very early Universe. Rife with speculation and liable to become largely irrelevant overnight, the last third of the book is the weakest section. But it is definitely worth reading, if only to find out how Kaluza, the founder of higher dimensional cosmology, learnt to swim. □

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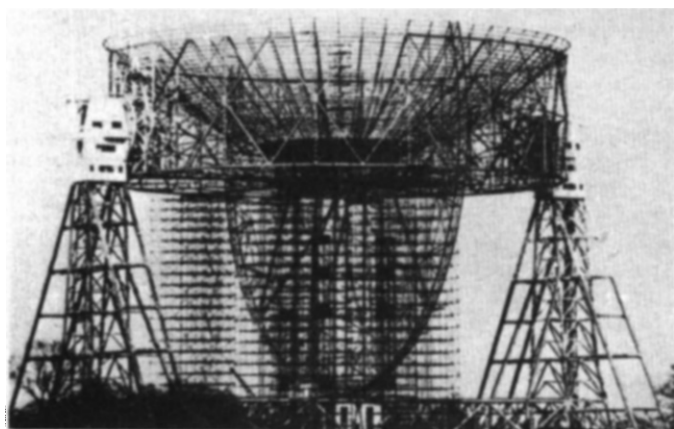
Fact or fiction?

C.E.M. Hansel

Science Confronts the Paranormal. Edited by Kendrick Frazier. Prometheus: 1986. Pp. 367. Pbk \$15.95, £12.95.

Science Confronts the Paranormal contains a selection of articles published by the Committee for the Scientific Investigation of Claims of the Paranormal in its journal *The Skeptical Inquirer*. The book is divided into two sections. The first of them, "Assessing Claims of Paranormal Phenomena", is mainly concerned with parapsychology — including poltergeists, and the claims made by mediums and professional "psychics" — together with palmistry and iridology. The second section, "Evaluating Fringe Science", covers unidentified flying objects, fringe archaeology, creationism, the Turin shroud, astrology and the Loch Ness Monster.

The general approach is set out in the introduction and early chapters. Stephen Toulmin suggests that consideration of the manner in which new ideas have been opposed will "induce a certain modesty even about our skeptical doubts". Paul Kurtz also considers that the claims made by "paranormalists" should not be rejected out of hand. But over the history of scientific inquiry it is difficult to identify a new idea that has been rejected for long, provided that observations could be con-



Jodrell Bank radio telescope nearing completion in April 1957. Taken from Bernard Lovell: A Biography by Dudley Seward, which includes a summary of Lovell's views on the nature and origins of the Universe. Now available in the USA (Salem House, \$21.95). For review, see *Nature* 312, 210; 1984.



The spoon-bending abilities of Uri Geller, a professional magician who claimed to be psychic, made headline news. Scientific tests of his powers have now become "little more than a collection of anecdotes"

firmed adequately or that a new hypothesis could be tested experimentally and confirmed by independent investigators.

Most of the claims examined in the first section are not new, but are dependent on ideas going back into prehistory that have failed to be accepted within science. They have not been totally rejected for scientists from many disciplines working within parapsychology have been attempting to provide repeatable experimental demonstrations of the various psychical phenomena for more than a century. When such a demonstration becomes available, it will then be necessary for the sceptics to see whether they can also confirm its findings. The main reason for examining such claims in advance of evidence supporting them is made clear by Kurtz: "... we are concerned not simply with paranormal beliefs in the laboratory but with their dramatization in the media".

Two types of investigation may be distinguished: first, experiments, in which the researcher decides on the experimental design and procedures to be adopted whilst the subject does as he is told; second, demonstrations, in which a person attempts to convince observers of his psychic powers. Here the investigator observes the subject while he performs under his own conditions, often reminiscent of those enjoyed by a conjuror in the music hall.

What has emerged from the experimental work is that attempts to confirm an original "positive" finding will either fail or "succeed" at a low level of significance, the result depending mainly on the experimenter. This has led to the conclusion within parapsychology that there is an "experimenter effect", which is not dependent on the slap-happy nature of the investigation but on some new interaction between the experimenter and the hypothetical process being considered. Of particular interest here is the discussion of an experiment on "remote viewing" — or clairvoyance — reported in *Nature* (252, 602; 1974) by Targ and Puthoff of the Stanford Research Institute (SRI). The experiment was shown by Marks and Kamman (*Nature* 292, 177; 1981) to con-

tain a schoolboy blunder that could account for the result. Marks himself reviews this and further experiments which claimed to indicate that the fault in the procedure made no difference to the result. His conclusion is that:

Well-controlled experiments never find the RV effect, while poorly controlled experiments nearly always do so. Data suppression, flawed methodology, and lack of replication lead to the conclusion that remote viewing is a cognitive illusion, an artefact of human error and wishful thinking.

An investigation of the second type is discussed by Martin Gardner in "How Not to Test a Psychic: The Great SRI Die Mystery". Uri Geller, a professional magician who claimed to be psychic, was tested during a visit to SRI where it was stated that he was able to name correctly the face uppermost on a concealed die on each of eight attempts and that he made no attempt on two occasions. After discussing means whereby any trained conjuror might have obtained similar results, and his attempts to find out more about the tests, Gardner writes:

What conclusion can we reach from all this? The most important is surely the following. What seemed to any reader of *Nature* to be a carefully controlled die test has now become little more than a collection of anecdotes.

The section on fringe science is concerned in the main with certain claims that are not inconsistent with contemporary scientific theory but that are not supported by evidence. A large amount of material is available here relating to matters that become headline news but are seldom reported fully and critically.

Science Confronts the Paranormal should be of considerable value to those puzzled by accounts of "paranormal" events appearing in the media, to teachers interested in the scientific method and to parents who are disturbed by what their children are taught. It could well be made compulsory reading for science correspondents and television producers. □

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Wide sweep

A.J. Thomson

Physical Methods for Inorganic Biochemistry. By John Wright, Wayne Hendrickson, Shigemasa Osaki and Gordon James. Plenum: 1986. Pp.384. \$71.40, £47.60.

THE task this book sets itself is ambitious. It attempts to cover the spectroscopic, diffraction and analytical methods used to probe the structural properties of the elements employed by living organisms. But, before beginning, the authors needed to decide whether to write a book about the methodology of the techniques, their principles and practice, or to describe the ways in which our understanding of biological systems has been advanced. This choice has not been made, and as a result most chapters fall short of meeting either goal.

The consequences of indecision are seen clearly in the chapter on the principles, practices and applications of nuclear magnetic resonance (NMR). Here the authors have slipped between three stools. Principles are stated briefly but inadequately, and it is doubtful that those readers who do not understand the basis of Fourier transform NMR, say, will be much clearer after reading this section. The description of practice extends to giving absurd detail for a circuit diagram of a low-pass noise filter — surely a topic for a technical manual — while applications are dealt with on an element-by-element basis; this makes a useful compendium, but hardly shows the powerful ways in which NMR has helped biologists.

To make things worse, the authors have tried to cover too many techniques, several of which are as yet of little established value in biological research. For example, nuclear quadrupole resonance (NQR) spectroscopy is an insensitive technique that has produced no information of biological significance. Electron spectroscopy (ESCA) is also out of place, whereas EXAFS (extended X-ray absorption edge fine structure) is not discussed even though it has had considerable impact. The sweeping scope has inevitably led to areas being tackled that are well outside the authors' competence — the statement that "there have been few temperature-dependent magnetic circular dichroism studies of transition-metal enzymes" is false and renders the chapter concerned out-of-date and inadequate.

Altogether, it is difficult to see the aim or the value of this book. Much of the material is available elsewhere, either in reviews or texts on the individual techniques written by more authoritative authors. □

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New frontier for stratigraphy

A.M.C. Şengör

The Geology of China. By Yang Zunyi, Cheng Yuqi and Wang Hongzhen. Oxford University Press: 1986. Pp. 303. £55, \$79.

WITH 9.6 million km² China covers nearly one-fifteenth of the world's land area and is the third largest country in the world. The geology of this vast portion of our planet is now being studied by some 70,000 geological personnel in China. Their results are reported regularly in some 50 Chinese periodicals and monograph series, only a few of which contain English abstracts. *The Geology of China*, the second book of its kind since Li Siguang's (J.S. Lee's) 1939 classic bearing the same title, has been written by two stratigraphers and a metamorphic geologist to provide an up-to-date survey of the subject. It is based mainly on the immense amounts of both published and unpublished work done since the foundation of the People's Republic.

The book contains a total of 20 chapters, divided into four main parts. Part I ("Background") briefly reviews the development of geology in China and the physical features of the country. Part II ("Stratigraphy") is the backbone of the book, consistent with the dominant interest of the authors. Here, in the first chapter the stratigraphic terminology employed in the book is reviewed and a synopsis of the stratigraphic evolution of China is presented. The next three chapters deal with the Archaean and the Proterozoic eons and the Sinian system. Nine of the remaining ten chapters of Part II treat individual systems from the Cambrian to the Cretaceous, while the last describes, too briefly perhaps, the Cenozoic. In each chapter sedimentary rocks are discussed in terms of stable (platform), mobile (geosynclinal) and transitional sedimentation types — this was an unfortunate choice, which makes understanding of former sedimentary environments in terms of present-day settings difficult. Although boundary and correlation problems are considered for each system, palaeobiogeographic provinces are touched upon only in chapters on the Carboniferous and the Permian systems.

Part III ("Magmatic and Metamorphic Rocks of China") contains two chapters, one each for the two major rock groups. Here the treatment is mainly regional and chronological.

In the last part ("Geotectonic Development of China"), two chapters are devoted to the tectonic framework and geotectonic units, and the geotectonic

development of China respectively. Although much lip service is paid to plate tectonics, and the few palaeotectonic cross-sections are drawn in basic plate-tectonic terms, the underlying framework in both of these chapters is based on an unhappy marriage between a Soviet-style fixist philosophy and plate tectonic mobilism. The descriptions and arguments are made even more difficult for the reader to follow because of the authors' peculiar employment of familiar terminology such as aulacogen and continental margin.

A serious limitation of the book is the almost complete lack of reference to foreign or cooperative research on Chinese geology. For example, Molnar and Taponnier's epoch-making studies on the neotectonics of China are not even mentioned. Further, there is no reference to the Franco-Chinese work on Tibet (absence of mention of the geophysical results is especially glaring), nor to the important magnetostratigraphic work by Liu Dongsheng and his co-workers on the loess deposits. Such omissions have in places led to some rather odd conclusions, such as the supposed late Mesozoic origin

and Cenozoic inactivity of the Altyn–Tagh fault.

The paucity of sketch-maps showing particular geological relationships and outcrops adds to the problem of comprehending both local and regional details, so the book is difficult to use to reinterpret the data in terms of models other than the authors'. Despite its deficiencies, however, *The Geology of China* is a must for those interested in the regional stratigraphy of this country. A subject and a stratigraphic index (both in Pinyin transliteration and in the original Chinese characters) increase the value of the book, as do the 19 black-and-white plates of fossils and photomicrographs of representative lithologies. However, readers would be well-advised to keep at hand either one of the post-1980 comprehensive editions of the *Times Atlas* or the *Zhonghua Renmin Gongheguo Fen Sheng Dituji*, for many of the places mentioned in the text are not on any of the maps in the book. □

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A diverse life

Colin Townsend

Community Ecology. Edited by Jared Diamond and Ted J. Case. Harper & Row: 1986. Pp. 665. \$37.50, £16.95.

SUPPOSE, for the sake of argument, that the genetic code, instead of being determined solely by DNA, was co-determined by seven classes of macromolecule. Suppose, too, that in a given species the relative role of the macromolecules varied with age, season, weather conditions and time since the last glaciation, and also tended to differ between large and small species, ectotherms and endotherms, and herbivores and carnivores. If this were true, we would surely not have our present complete understanding of the code. And yet, as the editors of this volume point out, this is exactly the problem that ecologists face in trying to explain how the abundances of interacting species in a community are co-determined by competition, predation, herbivory, disease, parasitism, mutualism and disturbance. Later in the book we are told that studying ecology resembles what it would be like to conduct a chemistry experiment if the chemist were only a few angstroms long and lived for only a few microseconds!

Attempts to solve the daunting problems of variability and scale in community ecology demand a diversity of methodologies to obtain the data and a diversity of models to interpret them. The methods

include laboratory experiments, field observations, experiments in the field, "natural" experiments involving comparative observations at several contrasting outdoor sites, and reconstructions based on historical records in library and museum archives and in fossil assemblages. All figure prominently in this stimulating collection of 33 essays by 35 authors (34 from North America and one from England). The book is written with the professional researcher in mind, but undergraduates will find much of the material accessible.

Each of the five sections of the book opens with a scene-setting chapter. The first, by Jared Diamond, compares the merits of the different experimental approaches and is followed by an account of probably the most ambitious laboratory experiment on community assembly yet attempted — Michael Gilpin and his co-workers describe how the results from 28 species of fruit fly studied singly can be used to predict, with surprising accuracy, the outcome of pair-wise interactions or ten species combinations.

Section 2 is concerned with species introductions and extinctions. Competition between species for food or space is potentially a potent force in the structuring of communities, with species co-existing only if they exhibit some minimal differences in their resource requirements. If this potential is realized (and there is much debate about the point), competition must operate through a process of selective extinction. Michael Moulton and Stuart Pimm have come up with a novel

analysis of historical data to address this question. Records of success or failure of over 100 introductions of exotic bird species to Hawaii during the past 125 years permit calculations of how a species' risk of extinction depends on the number and morphological closeness of other species in the community — a role for competition is indicated in this case.

Section 3 discusses the problem of the appropriate choice of spatial and temporal scale in investigations of community ecology, and Section 4 contrasts equilibrium and non-equilibrium theories of community ecology. A recurring theme in these sections, and elsewhere in the book, is the importance of historical data. Paradoxically, although those who study modern communities regularly debate whether communities are at equilibrium, rarely have they consulted the fossil evidence. Margaret Davis emphasizes that on any time scale from a decade to 100,000 years climate does not fluctuate about a mean value, but exhibits long-term trends. Her data for forest trees, and those of Thomas Van Devender derived from plant and animal fossil remains in packrat middens in the Chihuahuan Desert, emphasize how communities have frequently become dismantled and reshuffled as species shift their geographical location differentially in response to changing climate.

The final sections deal with the variety of forces structuring communities and with the variety of kinds of community that exist. The message is that there is no single model to describe all communities — some are structured mainly by competition, some by predation, some by unpredictable disturbances and so on — but neither is every community unique. Thomas Schoener makes an initial assault on the problem of defining a minimum number of properties of organisms and environments that will enable us to say, for example, that for organisms of Type A, in environments of Type B, the dominant structuring force(s) will be of Type C (D, etc.). But there is still a long way to go.

Thirty years ago, a book about the multi-species level of ecological organization would probably have dealt almost exclusively with the flux of energy and nutrients between the environment and the community. More popular today is an approach to the understanding of multi-species assemblages based on knowledge of the dynamics of the constituent species populations. Indeed, the old distinction between population ecologist (a student of the abundance and distribution of individual species populations) and community ecologist seems to be fast breaking down. □

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Bird cake

John Andrews

The Sparrowhawk. By Ian Newton. Poyser, Town Head House, Calton, Waterhouses, Staffordshire, UK/Buteo, PO Box 481, Vermillion, South Dakota 57069, USA:1986. Pp.396 + plates. £16, \$35.

IN THE battle being fought in many countries to protect rare or declining species, wildlife conservationists are often severely handicapped by lack of knowledge. What are the species' numbers and distribution? What are its requirements, and how can these be accommodated in a changing environment? Often the best we can do is to make an informed guess, always running the risk that error will harm the subject of our concern and weaken our modest influence still further. Against this background, Ian Newton's study of the sparrowhawk (*Accipiter nisus*) is valuable on two counts. First, it answers many of the questions relevant to the conservation of a bird much affected by human activities. Second, the research methodology and some of the conclusions may, with care, be applied to other accipiters and so advance research on them also.

Sparrowhawks breed in woods and forests across the Palearctic region from Ireland to Japan. In winter most of the boreal birds move south into the Middle East, India and south-east Asia. Close relatives occupy North America, Africa and southern Asia, the whole genus comprising about 50 species worldwide. Few escape the influence of mankind.

In Britain, progressive clearance reduced forest cover to 5 per cent of the land surface by the mid-nineteenth century, and accordingly restricted sparrowhawk distribution: then came the development of game preservation, and an era of intense persecution. Nonetheless, these evasive birds held on in small numbers, thinly but generally present over most of the country until, recently, organochlorine pesticides eliminated them from much of their range. Now, legal protection and, more importantly, changes in pheasant rearing methods have much reduced deliberate killing; afforestation has doubled the potential habitat and controls on organochlorines have permitted a rapid recovery in numbers over most of the country.

Newton, who is a senior ornithologist with the Natural Environmental Research Council, began his study of the species 14 years ago, selecting two areas of Scotland where he sought to trap and ring all individuals present and to find all their nests. From this basis he was able to explore the bird's ecology, making his own practical experiments to test hypotheses as the pro-

ject developed and drawing on other researchers' findings. The work was complicated (but made more rewarding) by the fact that male sparrowhawks are half the weight of the females, so that they differ greatly in habitat usage, range of prey taken and response to factors such as weather and inter-specific competition (not to mention being killed by their own fair sex!).

Many individual birds were studied throughout their lives, enabling Newton to provide here a detailed account of the species' hunting and prey selection, dispersal and migration, breeding mortality and population trends, and of the human impact on their well-being. Much of the material has already appeared in journals, but, like a cake, the book's ingredients gain from being mixed and cooked by a skilful hand. The text is supplemented by crisp monochrome photographs and line drawings, while copious figures present results graphically with the supporting data set out in appendices. There is a good bibliography and an extensive, helpful index.

Ian Newton has a well-deserved reputation for sound research and for presenting his results in a carefully reasoned and readable manner. *The Sparrowhawk* is well up to expectations; it will be enjoyed by amateur ornithologists and professionals alike. □

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**IMAGE
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The sooty owl from Birds of Eucalypt Forests and Woodlands: Ecology, Conservation, Management. Published by Surrey Beatty & Sons Pty, 43-45 Rickard Road, Chipping Norton, NSW 2170, Australia (A\$47, US\$43), it is based on the proceedings of a conference organized by the Royal Australasian Ornithologists Union.

The case for life as a cosmic phenomenon

from F. Hoyle and N.C. Wickramasinghe

The arguments in support of life as a cosmic phenomenon are not readily accepted by a culture in which a geocentric theory of biology is seen as the norm.

A LEADING article in a recent issue of *Nature* explained that we had ourselves to blame for experiencing difficulties of an unusual kind in the publication of referencing of our work¹. Our fault was that we had become caught up in the eccentric doctrine of panspermiology, a doctrine for which there was said to be little or no evidence. We have now been invited by the editor of *Nature* to explain why we differ from his judgement and why in our opinion there is indeed evidence in favour of our position. In our experience, people who become eccentric usually do so through a mental discontinuity, whereas we would claim to have progressed by a series of comparatively small steps over many years, with each step rather cautiously tested by either observation, experiment or calculation using generally accepted methods of deductive science.

Our ideas began in the middle 1950s with qualitative considerations on the nature of interstellar grains and with a straightforward generalization to interplanetary conditions of the well-known Urey–Miller prebiotic experiment². By the early 1960s we had added a refractory carbonaceous component to the ice-grain model of van de Hulst³ and had begun to calculate the extinction of starlight to be expected from complex mixtures of ice and carbonaceous particles^{4–6}. The correspondence between our results and the observed interstellar extinction of visible starlight was tolerable but not really good in view of the many adjustable parameters that were present in the calculations.

Eventually we discovered that appreciably better agreement could be obtained between observation and calculation provided the real part of the refractive index of interstellar grains was low, say 1.15, instead of being 1.3 as for ice or ~2.4 as for carbonaceous particles. A low refractive index turned out to be much better than the many-parameter adjustments we had formerly used. But what material could have a refractive index as low as 1.15? In seeking an answer to this question it was important that the grains be largely made up of cosmically abundant elements, because the amount of the observed extinction is so considerable that only abundant elements will suffice to give the necessary quantity of grains. Of materials based on common elements only solid hydrogen would have the required low refractive index it seemed. So for a while we investigated this possibility and

became enthusiastic about it^{7–9}. The trouble, however, turned out to be that solid hydrogen was too volatile and would evaporate away¹⁰. So it came about that we were left with an unsolved problem, and we had to put it aside for a decade until quite new ideas suggested themselves.

Organic polymers

From calculations made in 1968 of condensations occurring in large mass flows from astronomical objects we suggested that interstellar grains might contain a magnesium oxide–silicon oxide component¹¹ and when a so-called silicate emission-band near 10 μm in the infrared was observationally discovered the following year we had a new topic to become enthusiastic about^{12–16}. Unfortunately as it seemed at the time, our former disappointing experience repeated itself. Following an initial approximate success in matching the laboratory absorption properties of mineral silicate particles to the infrared observations¹⁶, the situation did not improve as better laboratory measurements and better observations were made. Discrepancies that were unsatisfactory remained, as they do to this day. It was with mounting frustration that we began to look through mountains of chemical literature, searching for a substance made-up of common atoms with infrared properties similar around 10 μm to the astronomical observations. It was from this intensive literature search that the idea at last dawned on us that organic materials in the form of polymeric structures might make up a major fraction of the interstellar grains¹⁷, and moreover that similar structures might be present in cometary dust¹⁸.

The step from inorganic to organic grains did not seem particularly revolutionary. Organic molecules in the gaseous interstellar medium had been discovered already by radioastronomers, although in nothing like the quantity of the grains, while as long ago as 1956 Platt had suggested the widespread presence of very tiny organic grains¹⁹. Still pursuing the infrared problem, we eventually found that among organic materials polysaccharides gave the best correspondence to the astronomical data^{20,21}, and it was at exactly this point in our work that we began to experience hostility from the referees of journals and from the assessors of grant applications at what was then the Science Research Council. We realize now that because

polysaccharides on the Earth are a biological product we had unwittingly made a contact that is deeply forbidden in our scientific culture, a contact between biology and astronomy. We did not see that we had sinned mortally and were deeply mystified since at first, we thought of polysaccharides as being produced abiologically. Nor did we realize that the cultural taboo that confronted us had already surfaced in 1870, only one year after the inception of this journal²¹.

Only gradually did we come to understand the difficulties of producing very large quantities of organic material by abiological processes²². The Fischer–Tropsch reaction, for example, which produces hydrocarbons from hydrogen and carbon monoxide depends on the use of carefully controlled catalytic surfaces, which would almost surely be quickly poisoned by corrosive sulphur compounds under cosmic conditions (it may be noted that the Fischer–Tropsch process could not be operated profitably to compete with natural oil at \$30 per barrel, even under the most expertly managed terrestrial conditions).

Biological solution

We are not sure of exactly what it was that caused biology to enter our perceptions, but we think it was noticing a series of drawings of a bacterium from which the internal water was progressively dried out. The cell wall remained in place as cavities developed in the interior, eventually yielding a particle of typically the size of an interstellar grain that was hollow. We realized at once that such a particle would behave with respect to visual light, to a close approximation like a particle of low refractive index, just the situation we had been seeking more than ten years earlier in the 1960s. We obtained an experimentally-determined size distribution of bacteria from a standard manual and were quickly able to calculate how such a size distribution of hollow particles would behave with respect to the extinction of starlight. The result was a truly excellent fit to the astronomical data, the data we had attempted to fit without real success by our many-parameter models in the 1960s²³.

On the strength of this result, Dr Shirwan Al-Mufti began to measure the infrared properties of a number of species of bacteria, finding to his and our surprise a remarkable constancy of absorption for

the wavelength range 3.3 to 3.5 μm , and a general constancy for the whole range from 3 to 4 μm (ref 24). If bacteria made up an appreciable fraction of the interstellar grains, as the observed extinction of visible starlight seemed to suggest, it was necessary therefore that the grains must possess the characteristic absorption pattern which Al-Mufti had measured in the laboratory. It happened not long after these experiments that the average absorption properties of grains along the whole path length from the galactic centre to the Earth was measured to a high degree of accuracy by Allen and D.T. Wickramasinghe²⁵. The results, made at no less than 60 wavelengths between 3 and 4 μm , agreed with the bacterial experiments to within an accuracy of typically about one small graticule division on a standard Perkin-Elmer pen chart, an accuracy that we, who had hoped for accuracy, found impressive²⁶.

We are aware that astronomers and chemists can be found who will claim that these results are not impressive, because equally good results could be obtained using plausible non-biological materials. Our answer is that equally good results have not been obtained using plausible non-biological materials. Such claims are advanced and listened to only because they are designed to be culturally acceptable, whereas our results, although based on careful observations, experiments and calculations are not culturally acceptable. In such a situation the critic is permitted to say anything at all without being weighed in the balance and found wanting.

Stellar replication

An attempt is often made to saddle us with the criticism that bacteria could not replicate in interstellar space. We have never said that they did. Our model is a cyclic one, with bacteria replicating as a by-product of star formation in environments where replication *can* occur²⁷⁻²⁹. Following star formation, a fraction of the bacteria produced are expelled into the interstellar medium. Some die, some survive — quite likely only a minority survive — to become incorporated in further star formation processes, and so on around the cycle. Biological replication is an exponential process that can immensely outstrip all abiological processes. Granted sustained conditions for the growth of a bacterial culture, a single viable bacterium could grow to a mass of bacteria equal to the Earth in about nine days, a mass equal to the galaxy in about fifteen days, and a mass equal to the whole visible universe in about twenty days.

It is a necessary corollary that bacteria must be space-hardy, unless after arriving here on Earth mutations have destroyed properties which they possess initially^{29,30}. A viable strain of *Streptococcus mitis* was recovered after two years of exposure to

conditions on the surface of the Moon³¹. Bacteria can be taken down to near zero pressure and temperature without loss of viability, provided suitable care is exercised in the experimental conditions^{32,33}. Bacteria can survive after exposure to pressures as high as 10 tonnes cm^{-2} (ref. 34) and after flash heating under dry conditions at temperatures up to 1,000 K (ref. 35). Viable bacteria have been recovered from the interior of an operating nuclear reactor³⁶. A fraction of bacteria remain viable even after extremely heavy flash doses of high energy radiation, upwards of a megarad, while it seems that bacteria can repair themselves continuously in a maintained environment of high radiation intensity, to the extent of repairing tens of thousands of breaks in their nucleic acid structure^{37,38}. These are not properties one would have expected to evolve on the Earth, but they are all properties necessary for survival in space. Damage from ultraviolet light, which is often raised as a problem is actually no problem, because ultraviolet light is easily shielded against^{29,39}.

An individual comet has only a small mass, but if one considers all the comets that are believed to exist in the present-day Oort cloud, and to have existed over the whole history of the Solar System, their combined mass could well be comparable to that of the outer planets Uranus and Neptune, about 10^{29} grams. If the $\sim 10^{11}$ stars of our Galaxy are typically endowed with similar quantities of cometary material, the total cometary mass for the whole Galaxy would be $\sim 10^{40}$ grams, very close to the mass of the interstellar grains.

We were led from this consideration to think of a swarm of comet-like bodies, present towards the outer regions of the Solar System in its early history, and being made warm in their interiors by radioactive heat, as the likely sites for early biological replication⁴⁰⁻⁴². As we put it on one occasion, such an individual cometary interior would be like a vast laboratory with a floor area of some thousand square kilometres and with a height comparable to that of the Empire State Building. If other dwarf stars are mostly like the Solar System, there could be more than 10^{20} such laboratories in the Galaxy, which makes it only a matter of commonsense we believe to regard such bodies as a more favourable venue for the development of life than the initially sterile surface of a small planet like the Earth, which even today has a biosphere with a mass of not more than $10^{18} - 10^{19}$ grams.

Holding these views, it was easy for us to predict that an important fraction of the dust expelled from comet Halley would be organic in composition, that bacteria in the dust would become hollow as water within them dried out, thereby yielding particles with an average mass density less than 1 gm cm^{-3} , and that organic material

at the surface of comet Halley would be dark⁴³. We have heard astronomers speaking of the surface material of comet Halley as being dark like tar, perhaps without realizing that tar is itself a biological product.

It has often been suggested to us that our views might be tested by a suitable satellite experiment. Such an experiment would be expensive and difficult because of high impact speeds, as we saw in the recent Giotto encounter. Another suggestion often made is to send balloons or rockets into the high atmosphere with the aim of recovering viable bacteria from space. This has actually been done, repeatedly. On every occasion viable bacteria were obtained⁴⁴⁻⁴⁸, and on every occasion the result was discounted because of the possibility, admittedly serious in some cases, of terrestrial contamination. When a result is culturally unacceptable it will always be discounted on some excuse or other. One cannot win that way.

The Earth is perpetually embedded in a halo of cometary material, of which some 1,000 tonnes enter the terrestrial atmosphere each year. Because of the low density of the upper atmosphere, incident small particles of the sizes of bacteria and viruses could land 'soft' without viability being destroyed by flash heating. After intervals ranging from months to years such particles eventually settle to ground level, where they would be added to the already-existing reservoir of bacteria and viruses. It is to the potential interaction of such incoming particles with plants and animals that one can best look for a direct verification of these ideas. For some years we have been much concerned with this interesting and informative mode of verification^{22,41}. It is our opinion that a large body of facts exists to prove the correctness of the general picture outlined above. The topics themselves being in the medical field of epidemiology, would take us far away from the rest of the present article. Besides which, we are about to run out of the space the Editor has kindly allotted us, and so must refer the reader, if any should be interested, to a recent publication entitled: *Viruses from Space*⁴⁹. We have found it not a little odd to find our views taken more seriously, or at least received more politely, by the medical profession than they have been by astronomers and chemists, for whom we retain not a few rods in pickle. □

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ARTICLES

Detection of an X-ray-ionized nebula around the black hole candidate binary LMC X-1

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Optical spectra of the black hole candidate X-ray binary LMC X-1 reveal that the system is surrounded by an extended, highly ionized He III region, N159F, which appears to be the long-sought-for example of an X-ray-photoionized nebula. The spatially resolved temperature and ionization structure allows us to measure the previously unobservable extreme ultraviolet (EUV) luminosity of an accreting compact object.

DESPITE a fairly accurate (± 3 arc s) Einstein HRI (high-resolution imager) position for the bright (X-ray luminosity $L_X = 2 \times 10^{38}$ erg s $^{-1}$) variable X-ray source LMC (Large Magellanic Cloud) X-1 (ref. 1), neither of the previously suggested optical counterparts $^{2-4}$ can be excluded on positional ground alone (Fig. 1). The optical properties of the B5 I star R148 ($V = 12.2$ mag) appear normal for its spectral type, including the slight radial velocity and photometric variations 5,6 which are observed in most supergiants. More promising for an optical identification is the fainter ($V = 14.5$) O7 III-type star '32', which reveals the variable He II $\lambda 4,686$ and $\lambda \lambda 4,640-4,650$ emission $^{4-6}$ generally considered to be the hallmark of high-luminosity massive X-ray binary (MXRB) optical counterparts. Hutchings *et al.* 6 have suggested that part of the 4,686-Å emission stems from the surrounding H II region N159F (refs 7, 8; see also Fig. 1); but these authors did not elaborate on this potentially important result.

In order to study the reported radial velocity variations 6 in star 32 which suggest that LMC X-1 might be a black hole (a conjecture which is supported by the interpretation of its extremely soft X-ray spectrum 9), and to investigate possible X-ray heating effects on the H II region N159F, we have obtained long-slit spectra of this region. Our observations reveal a remarkable ionization structure, suggesting that we have observed the

first example of a spatially resolved X-ray-ionized nebula.

Observations

We carried out two-dimensional charge-coupled device (CCD) spectrophotometry 4 nights in December 1984, using the B&C spectrograph attached to the 3.6-m ESO telescope at La Silla, Chile. Here we are concerned mainly with the results of our observations on 21 December, which were obtained with a reciprocal dispersion of 114 Å mm $^{-1}$, resulting in a full width at half-maximum (FWHM) resolution of 5 Å in the wavelength range 3,600–5,200 Å. The relatively large width of the slit (2 arc s) made this configuration most suitable for the study of nebular emission from the surrounding H II region N159F. The slit orientations were chosen as shown in Fig. 1b: A, including both candidates R148 and star 32 and B, roughly perpendicular to the former orientation, including star 32. Two longer-wavelength CCD spectra which also include the 5,000–6,700 Å region, at a lower resolution of 8 Å, were obtained in May 1985.

The images were flat-field- and extinction-corrected and calibrated in wavelength and flux from the observation of a He-Ar lamp and the spectrophotometric standard stars LLT1020, EG21 and L745-46A, respectively 10 , using the image processing facilities IHAP and MIDAS at ESO, Garching.

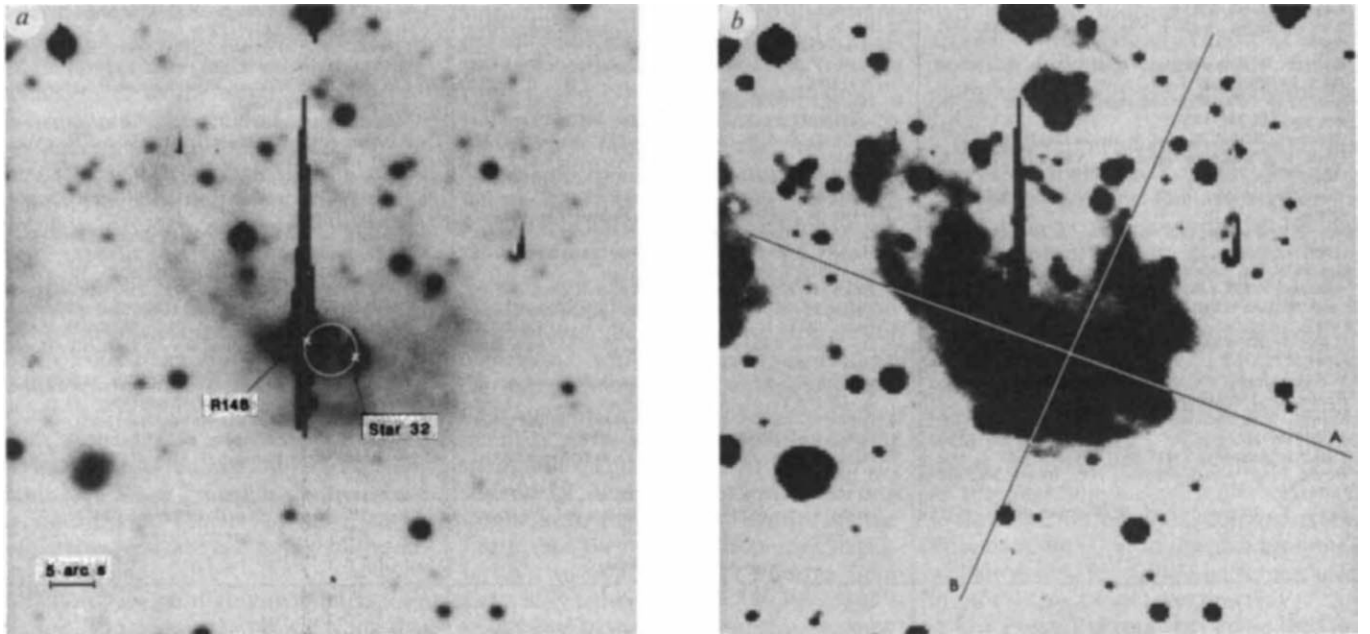


Fig. 1 Direct CCD images of N159F obtained with a Gunn *r* filter, emphasizing nebular H α emission. The HRI error circle (radius ~ 3 arc s) of LMC X-1 includes the 12.1-mag B5 I star R148 (strongly overexposed and elongated due to charge transfer effects from the read-out process of the CCD chip) and the presumed O7-III-type optical counterpart (star 32). The image of another fainter star (17 mag) can be seen close to R148. *a* and *b* were obtained from the same image with different high and low cuts, in order to emphasize the presence of stars (*a*) and nebular emission (*b*). The orientations of the slit in scans A and B are shown in *b*.

From the reduced images we measured the intensity of several nebular emission lines as a function of position along the slit. As suggested from inspection of Fig. 2, most emission lines are strongly enhanced in the vicinity of LMC X-1. H β , for instance, rises steeply from a background intensity of ~ 10 to a maximum value of 75 in units of 10^{-16} erg cm $^{-2}$ s $^{-1}$ arc s $^{-2}$ (Fig. 3). The extent of ~ 40 arc s (corresponding to 10 pc at the distance of the LMC) and total flux $F_{\beta} = 10^{-11}$ erg cm $^{-2}$ s $^{-1}$, corrected for interstellar extinction with optical reddening $E(B-V) = 0.37$

(ref. 8), can be accounted for by a Strömgren-sphere model of uniform electron density ($N_e = 40$ cm $^{-3}$), excited by the ultraviolet flux of an O7 star. Note that the B5 star R148 should not produce a substantial flux of H Lyman-continuum photons.

The [O III] emission is even more enhanced near LMC X-1. The [O III]($\lambda 4,958 + 5,007$)/H β line ratio, which is sensitive to the degree of excitation, peaks at 7.5 ± 0.2 , compared with a value of ~ 2.5 more than 20 arc s away (Fig. 4). The electron temperature derived from the $\lambda 4,363/\lambda (4,958 + 5,007)$ intensity ratio¹¹ around LMC X-1 is 13,500 K, which is $\sim 5,000$ K higher than in the more distant parts of the H II region.

Table 1 lists additional emission line intensities measured close to LMC X-1 and ~ 1.3 arc min away (positions 1 and 2, respectively; see Fig. 3a). Note the increasing ionization towards LMC X-1, as revealed by the [O II] $\lambda \lambda 3,726-3,729$ /H β and [Ne III] $\lambda 3,868$ /H β line ratios at positions 1 and 2. To produce the high nebular excitation, the effective stellar temperature should exceed $\sim 45,000$ K (refs 12, 13); however, this is inconsistent with the $T_{\text{eff}} = 35,000$ K determined from the IUE (International Ultraviolet Explorer) study of star 32 (ref. 8).

The most convincing evidence for the presence of a source of high excitation is provided by our detection of extended He II $\lambda 4,686$ emission ('He III region', Figs 2, 5) with a diameter of 24 arc s, corresponding to 6 pc in the LMC. The formation by recombination of doubly ionized helium requires a substantial intensity of He $^{+}$ Lyman-continuum photons ($h\nu > 54$ eV), which cannot be provided by even the hottest normal O-stars, as shown by the observation that this transition is never present in the spectra of normal H II regions but can be quite strong in planetary nebula (PN) with central star temperatures of $\sim 10^5$ K.

A PN-type nature of the He III region is, however, ruled out on the basis of the extreme population I environment in N159F and the large extent compared with the diameters of PN in the LMC, which do not exceed 4 arc s (ref. 14). Collisional ionization and excitation in a supernova remnant (SNR) shock can most probably also be excluded, as there is no indication of extended X-ray emission from LMC X-1¹⁵. Furthermore, our red spectra (see Table 1) do not show enhanced forbidden lines

Table 1 De-reddened line strengths relative to H β (=100)

Emission line	Position 1	Position 2
[O II] 3,727 Å	285	391
[Ne III] 3,868	42	8
H δ 4,101	24	25
H γ 4,340	48	48
[O III] 4,363	7	1.0
He I 4,471	2	4
N III/C III 4,640–4,650	<1	<1
He II 4,686	10	<0.5
[Ar IV] 4,711	2	<1
H β 4,861	100	100
[O III] 4,959	171	49
[O III] 5,007	538	157
He I 5,876	10	10
[O I] 6,300	8	4
[O I] 6,364	2	2
[N II] 6,548	8	10
H α 6,563	286	286
[N II] 6,584	29	34
[S II] 6,717*	32	34
[S II] 6,731*	23	24
C(H β)	0.582	0.302

Probable errors in the relative intensity of strong lines ($I_{\lambda}/I_{\beta} \geq 8$) are dominated by the relative uncertainty in our flux calibration ($<10\%$). The accuracy of weaker intensities is limited by photon noise and reaches $\pm 40\%$ for the faintest detected emission.

* Values obtained from observations with the 2.2 m telescope.

Table 2 Observed parameters for the X-ray-ionized nebula N159F around LMC X-1

X-ray spectrum	$L_{1-10 \text{ keV}} = 2 \times 10^{38} \text{ erg s}^{-1}$; thermal bremsstrahlung, $kT = 2 \text{ keV}$, $N_{\text{H}} = 9 \times 10^{21} \text{ cm}^{-2}$
Stellar spectrum	O7, $T_{\text{eff}} = 37,000 \text{ K}$, $M_V = -5.0$, $E(B-V) = 0.37$
Ambient electron density	$N_e = 40 \text{ cm}^{-3}$, probably smaller near LMC X-1
H β luminosity	$L_{\text{H}\beta} = 3.2 \times 10^{36} \text{ erg s}^{-1}$
He II $\lambda 4,686$ luminosity	$L_{4,686} = 1.5 \times 10^{35} \text{ erg s}^{-1}$
H II region radius	$r_{\text{H II}} = 5-6 \text{ pc}$
He III region radius	$r_{\text{He III}} = 3 \text{ pc}$
O III electron temperature	$T_e = 13,500 \text{ K}$ in the He III region 9,000 K outside

due to low-ionization species such as O I, S II and N II often exhibited by SNRs¹⁶. Also, the $\lambda 4,686$ emission has the same (instrumental) width of 170 km s^{-1} (FWHM) and radial velocity of $261 \pm 10 \text{ km s}^{-1}$ (derived from our higher-resolution spectra) as the lower-excitation lines, suggesting that it is not produced in the high-velocity knots observed in many SNR¹⁷. Rejecting a PN or SNR nature, we are left with the hypothesis that LMC X-1 is responsible for the formation of the He-III region in N159F.

He II $\lambda 4,686$ photon counting

Before discussing our findings in the context of increasingly detailed models for X-ray-ionized nebula¹⁸⁻²², we take advantage of the 'photon counting' property of the He II $\lambda 4,686$ line in order to measure the hitherto unobservable EUV flux from LMC X-1.

Although our spectrophotometric mapping of N159F is not complete we can reasonably estimate a total flux $F_{4,686} = 1.2 \times 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$ in the $\lambda 4,686$ line, assuming that our scans are representative of the spatial distribution of the emission. Correcting for interstellar absorption towards LMC X-1⁸ and taking 52 kpc for the distance to the LMC, we find (within a factor of 2) an emission-line luminosity $L_{4,686} = 1.5 \times 10^{35} \text{ erg s}^{-1}$, this being proportional to the absorption rate of He⁺ Lyman-continuum photons, Q'_4 , in the nebula:

$$Q'_4 = \int_{54 \text{ eV}}^{\infty} L_e / \epsilon (1 - \exp(-\tau_e)) d\epsilon$$

$$= \frac{L_{4,686}}{h\nu_{4,686}} \frac{\alpha_B(\text{He}^+, T)}{\alpha_{4,686}^{\text{eff}}(T)} = 2 \times 10^{47} \text{ s}^{-1} \quad (1)$$

where ϵ is the photon energy, L_e/ϵ is the photon number spectrum of LMC X-1 (emitted photons $\text{s}^{-1} \text{ keV}^{-1}$), $\alpha_B(\text{He}^+, T)$ the recombination coefficient summed over all levels above the ground state and $\alpha_{4,686}^{\text{eff}}(T)$ the effective recombination coefficient for the emission of He II $\lambda 4,686$ photons (luminosity $L_{4,686}$), carrying an energy $h\nu_{4,686}$. Note that the ratio of these

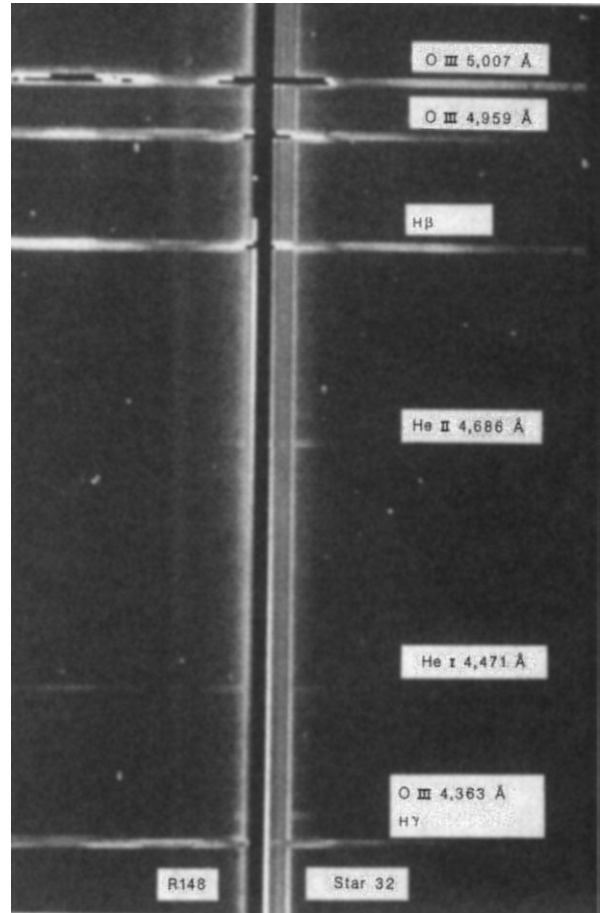
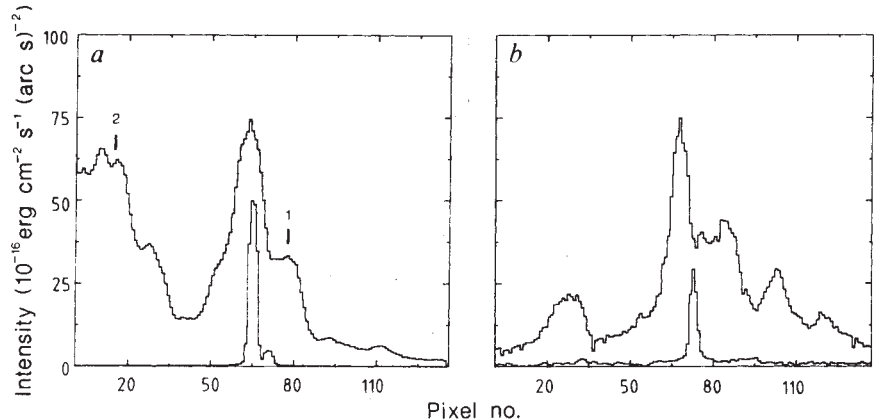


Fig. 2 A section of the 114 Å mm^{-1} two-dimensional spectrum (scan A) with the slit orientated in order to include R148 and star 32. The stellar continua appear as black (R148) and grey (star 32) vertical bands in this representation. The high and low cuts were chosen so as to emphasize faint nebular emission. Note in particular the extended distribution of He II $\lambda 4,686$ emission which peaks at the position of star 32. One pixel corresponds to $1.17 \text{ arc s} \times 2 \text{ Å}$.

coefficients (~ 5.2) depends only weakly on the electron temperature.

The optical depth due to He⁺, $\tau_e = \int_0^\infty \sigma_e N_{\text{He}^+} ds$ (where s is distance and σ_e is the photoionization cross-section of He⁺, which decreases as ϵ^{-3} above the threshold of 54 eV) can be estimated by noting that He⁺ is the prevailing ionization stage outside the central parts of the He III region. This is shown by the fact that the (reddening-corrected) He/H recombination line ratio here, $I_{4,471}/I_{\beta} = 0.030 \pm 0.002$, implies a He⁺ abundance of

Fig. 3 Intensity of nebular H β emission as a function of position along the slit (a, scan A; b, scan B). The nearby continuum flux has been normalized appropriately and subtracted to correct for the contribution of the stellar emission and H β absorption. The continua due to R148 and the 10 times fainter star 32 (scan A) and star 32 (scan B); which were scaled down by arbitrary factors, are shown in the lower curves to display their position with respect to the nebular emission along the slit. Note the strong enhancement of the emission near the star over a background level of $15 \times 10^{-16} \text{ erg cm}^{-2} \text{ s}^{-1} (\text{arc s})^{-2}$. Marks 1 and 2 show positions where the nebular spectra summarized in Table 1 were extracted. 1 pixel = 1.17 arc s .



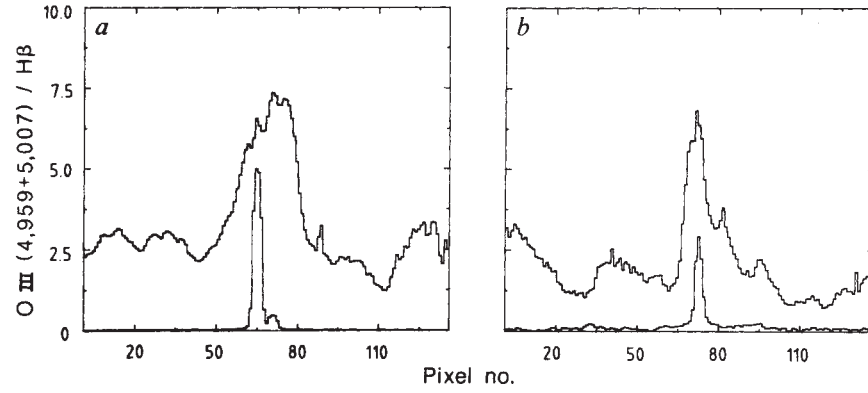


Fig. 4 The [O III] ($\lambda\lambda 4,959 + 5,007$)/H β line ratio as a function of position along the slit (*a*, scan A; *b*, scan B). The lower curves are scans of the stellar and nebular continuum. The maximum value of 7.5 is reached near the position of star 32. 1pixel = 1.17 arc s.

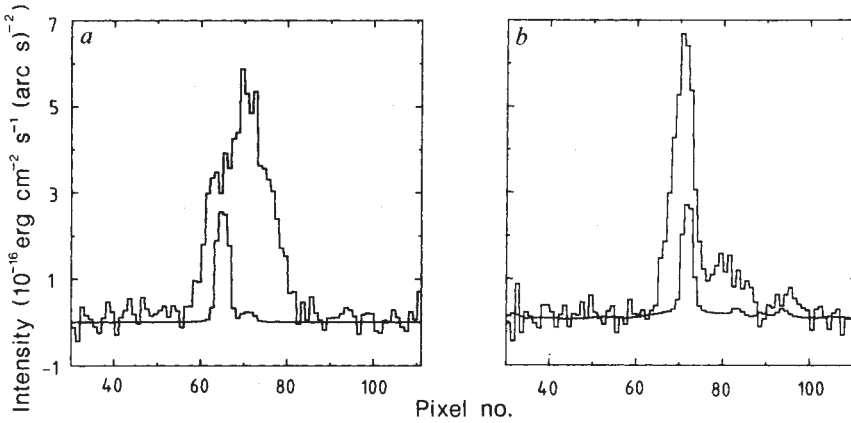


Fig. 5 The He II $\lambda 4,686$ intensity as a function of position along the slit (*a*, scan A; *b*, scan B). The lower curves are scans of the stellar and nebular continuum. There is an unresolved component present due to stellar emission from star 32. The extended emission has a diameter of 24 arc s (He III region), corresponding to 6 pc at the distance of the LMC and is centred within 1 arc s on star 32.

0.070 ± 0.005 , which is in fair agreement with the total average He abundance of 0.085 measured in LMC H II regions²³. A shell with a thickness of 3 pc and a He⁺ density of 3 cm^{-3} has an optical depth of ~ 40 at 54 eV. The nebula becomes optically thin, however, for He⁺ Lyman-continuum photons above $\epsilon_u \approx 0.2\text{--}0.3$ keV. Accordingly, we can approximate Q_4 by

$$Q_4 = \int_{54 \text{ eV}}^{\epsilon_u} L_e / \epsilon \, d\epsilon \quad (2)$$

provided that the intrinsic spectrum does not fall off too rapidly below ϵ_u . At higher photon energies, K-edge photoionization of heavier elements, notably of oxygen at 0.53 keV, is the main opacity source, but these photons are less important for the He ionization balance.

The X-ray spectrum of LMC X-1, which has been observed between 0.6 and 20 keV with the HEAO-1 (High Energy Astrophysical Observatory) A2 MED and HED and Einstein SSS experiments⁹ and, coordinated with our optical investigation, with the Exosat observatory (S. Ilovaisky, personal communication), exhibits relatively strong low-energy absorption with an equivalent column density N_H of $(9 \pm 2) \times 10^{21}$ equivalent H atoms cm^{-2} . Combining the column density with the optical reddening, $E(B-V) = 0.37$ (ref. 8), we obtain the ratio $N_H/E(B-V) = (2.4 \pm 0.6) \times 10^{22}$ H atoms $\text{cm}^{-2} \text{ mag}^{-1}$, which is four times higher than the galactic value but agrees well with the LMC gas to dust ratio deduced from interstellar Lyman- α absorption profiles²⁴.

We note that the contribution from the H II Strömgren sphere ($r = 5$ pc, $N_e = 40 \text{ cm}^{-3}$; see Table 2) amounts to only 7% of the total equivalent column density, implying that most of the X-ray and optical absorption is due to an extended neutral envelope, visible in 21-cm surveys, with a total column density of 6×10^{21} H atoms cm^{-2} (ref. 25).

Assuming that the $kT = 2$ keV thermal bremsstrahlung model which represents a satisfactory fit to the Exosat ME (0.7–10 keV) observations of LMC X-1 ($L_e = 1.4 \times 10^{38} g_R(\epsilon) \exp(-\epsilon/2 \text{ keV})$ erg $\text{s}^{-1} \text{ keV}^{-1}$, where g_R is the Gaunt factor²⁶; S. Ilovaisky, personal communication) can be extrapolated into the EUV,

and anticipating that photoelectric absorption between the X-ray source and the nebula is negligible (see below), we can integrate the differential photon rate L_e/ϵ to find (from equation (2)): $Q_4 = 3(4) \times 10^{47}$ photons s^{-1} for $\epsilon_u = 0.2$ (0.3) keV. The close agreement, within a factor of 2, with the value of Q_4 derived from He II $\lambda 4,686$ photon-counting arguments supports the validity of our approach, at least to a first approximation.

Because a power-law model with an energy spectral index $\alpha = 1.7 \pm 0.1$ in the range 0.6–3 keV resulted in a substantially better fit to the Einstein SSS data⁹, we have also investigated how far this model might be valid at lower energies without violating our photon-counting constraint (equation (2)). If the power law extended down to 54 eV, we would expect a $\lambda 4,686$ flux 50 times larger than actually observed. In order to produce the observed $\lambda 4,686$ luminosity, the intrinsic X-ray spectrum from LMC X-1 should either be cut off below 0.2 keV or substantially flatten ($\alpha < 0$) between 0.5 and 0.3 keV. The latter alternative qualitatively agrees with the ‘cool disk’ model²⁷ proposed to explain the spectra of accreting black holes in their high state.

Are the derived He⁺ Lyman-continuum flux from LMC X-1 and the ambient gas density consistent with the observed extent of the He III region? In the optically thin case the radius r_R of this region can be estimated by equating the rate of photoionization and recombination:

$$r_R = \left(\frac{L_X \gamma}{4\pi N_e \alpha_B(\text{He}^+, T)} \right)^{1/2} \quad (3)$$

where γ is the photoionization rate coefficient normalized to unit source intensity²¹:

$$\gamma = L_X^{-1} \int_{54 \text{ eV}}^{\infty} L(\epsilon) \sigma(\epsilon) \frac{d\epsilon}{\epsilon} \quad (4)$$

In the optically-thick limit, recombination occurs roughly at the Strömgren radius:

$$r_S = \left(\frac{3Q_4}{4\pi N_{Hc}^{++} N_e \alpha_B(\text{He}^+, T)} \right)^{1/3} \quad (5)$$

Inserting the observed parameters from Table 2, we find $r_R \approx r_S \approx 2-3$ pc, in good agreement with the radius of the observed He II $\lambda 4,686$ emission line region.

Here we have assumed that the He III region is observed in a static situation; that is, that the ionization front moving as $r(t) = r_{eq}(1 - \exp(-t/\tau_{He II}))^{1/3}$ (where $\tau_{He II} = (N_e \alpha_B(H_e^+, T))^{-1}$ is the He^{++} recombination timescale) has reached its equilibrium extent, r_{eq} . $\tau_{He II}$ (≈ 400 yr for $N_e = 40 \text{ cm}^{-3}$) therefore sets a lower limit to the time LMC X-1 has been X-ray active, and provides the timescale with which the structure of the He III region will react to variations of X-ray input from LMC X-1.

In order to estimate the fraction of doubly ionized He, we use the He II $\lambda 4,686/H\beta$ recombination line ratio, $I_{4,686}/I_\beta$, which reaches a maximum value of 0.095 ± 0.010 (corrected for stellar contribution and reddening) around star 32.

Using

$$\frac{\int N_{He^{++}} N_e ds}{\int N_H^+ N_e ds} = \frac{I_{4,686}}{I_\beta} \frac{\alpha_{\beta}^{eff}(T)}{\alpha_{4,686}^{eff}(T)} \frac{h\nu_\beta}{h\nu_{4,686}} \quad (6)$$

and a He abundance of 0.085 (ref. 23), we find that, averaged over the line of sight towards LMC X-1, the ionization fraction amounts to $N_{He^{++}}/N_{He} = 0.10 \pm 0.01$. Even taking into account that the H II zone around star 32 is 2-3 times larger than the $\lambda 4,686$ emission line region, and allowing for a background contribution from N159F of 30% to the observed $H\beta$ intensity, we find that He appears not to be fully ionized ($N_{He^{++}}/N_{He} \approx 0.4$) in the inner parts of the He III region, unless the density there is lower than in the ambient H II region. A decreasing density and the increase of temperature towards the X-ray source suggests an isobaric, rather than constant-density, cloud (see below).

Keeping in mind the uncertainty involved in the measurement of the de-reddened $\lambda 4,686$ flux, the fact that the extent of the He III zone reflects a time average of the X-ray luminosity, and that we did not take into account the diffuse radiation field from recombinations and transitions of heavy elements, we feel that our modified 'Zanstra temperature' argument provides a first clue to the otherwise unobservable EUV part of the emission from an accreting compact star.

So far we have not fully exploited the information provided by our spatially resolved spectroscopy concerning the ionization and temperature structure of the nebular region around LMC X-1. First, we note that the centre of gravity of the He II $\lambda 4,686$ intensity profile in scan A (Fig. 5) coincides within 0.6 arc s with star 32. Here we have discarded the unresolved stellar component, which contributes $\sim 10\%$ to the observed flux. Scan B exhibits a more strongly peaked $\lambda 4,686$ profile near star 32, with lower-intensity emission ~ 10 arc s to the north. Again, the centre of gravity of the extended emission deviates by < 1 arc s from the star. We take this as convincing evidence that star 32 is the optical counterpart of LMC X-1.

A second point concerns the presence of a relatively strong nebular continuum in N159F. Here we find that the intensity in the continuum, I_ν (measured near $H\beta$), closely follows the variation of I_β along the slit (see Fig. 3), and has an I_ν/I_β ratio of $(5.0 \pm 0.6) \times 10^{-14} \text{ Hz}^{-1}$, which is ~ 8 times higher than the value calculated from atomic processes (free-free, bound-free and two-photon emission²⁸) alone. This result implies an intimate mixture of gas and dust in N159F, which scatters stellar radiation from star 32 (and possibly R148) into our line of sight and also explains the extended ultraviolet continuum emission observed with the IUE around LMC X-1 (ref. 8).

Comparison with models

Drawing on early studies of the physical conditions prevailing in a gas cloud around a strong X-ray source^{18,19}, several investigators have extended the calculations by including important effects such as Auger emission, Compton heating and charge-transfer reactions. The most extensive calculations were per-

formed by Kallman and McCray²¹ and Kallman²², who explored the nebular response to several X-ray input spectra, assuming constant gas density and constant pressure clouds.

For a given X-ray spectrum the ionization and temperature structure depend only on the ratio of X-ray photon flux to particle density, $\zeta = L_X/nR^2$ (ref. 18), where n is the particle density at a distance R from the source, provided that the cloud is optically thin. Radiative transfer effects become important for larger values of the column density scaling parameter $(L_X n)^{1/2}$ (ref. 21), starting at low photon energies where the opacity is greatest. As shown above, N159F has an optical depth of 40 at the He^+ ionization threshold, which then quickly drops to values < 1 for photons with $\varepsilon > 0.2-0.3$ keV.

Model 2 of ref. 21, which assumes a thermal-bremsstrahlung-type spectrum with $kT = 10$ keV, a luminosity $L_X = 10^{37} \text{ erg s}^{-1}$ and a constant ambient density of 10^3 cm^{-3} , has about the right column density scaling parameter to be compared with the observed X-ray heating and ionization effects around LMC X-1.

Helium is expected to be doubly ionized ($He^{2+}/He > 0.9$) for $\zeta > 5$ (in cgs units). For increasing distance from the source, different ionization stages coexist, decreasing the relative abundance of He^{++} to $< 10\%$ for $\zeta < 0.4$. The low efficiency of high-energy photons for the complete ionization of helium is clearly demonstrated by the model calculations of ref. 20 which assume input spectra with a low-energy cut-off at 1 keV. Here, He^{++}/He drops below 0.1 for $\zeta < 100$, implying a He III region which is 16 times smaller than in model 2 of ref. 21. At the same total luminosity the soft ($kT = 2$ keV) spectrum of LMC X-1 provides ~ 5 times more flux below 1 keV than the $kT = 10$ keV model; therefore, we consider an equivalent spectrum for LMC X-1, with $L_X = 10^{39} \text{ erg s}^{-1}$ and $kT = 10$ keV.

At an ambient density $N_e = 40 \text{ cm}^{-3}$ (Table 2), ionization parameters of 0.4 and 5 translate into distance of 2.6 and 0.7 pc from the X-ray source. The former radius closely agrees with the observed extent of the He II $\lambda 4,686$ emission and with the result of the previous estimate on the basis of simple recombination and Strömgren-sphere arguments.

Moving inward from $\zeta = 0.4$ to 5, the temperature increases from 1×10^4 to 1.8×10^4 K. Our O III observations qualitatively support this type of temperature structure, with T_e increasing by $\sim 5,000$ K from the outer to the inner parts of N159F (Table 2).

For a more quantitative comparison, one must take into account the distribution of O^{2+} ions within the nebula, which strongly deviates from that in model 2 due to the presence of the stellar ultraviolet continuum from star 32. Here the stellar radiation field keeps oxygen doubly ionized throughout the H II region (see Fig. 4), and therefore strongly weighs the observed [O III] line ratio towards lower temperatures.

The presence of the ultraviolet continuum from star 32 has another important effect on the ionization structure of N159F: it removes the H I and He I atoms, thus suppressing charge-transfer reactions. It has been argued²⁹ that this effect increases the column densities N_i^j of several multiply ionized trace elements by factors of 3 to 25, and moves the location of the shell in which a given ion is found outward by a factor of 1.4-6 in radius.

Using the scaling laws $N_i^j \propto (L_X n)^{1/2}$ (ref. 21) and taking into account the observed abundance in LMC H II regions²³, we can predict interstellar column densities of 5×10^{15} , 5×10^{14} and $8 \times 10^{15} \text{ cm}^{-2}$ for the ions C IV, N V and O VI, respectively, which have resonance lines in the observable ultraviolet. These values are 1-2 orders of magnitude larger than those in the line of sight towards the galactic massive X-ray binaries HD153919 (ref. 30) and HD77581 (ref. 31) and should therefore be easily detectable in future high-resolution ultraviolet spectroscopy of LMC X-1.

As there is observational evidence for increasing temperature and decreasing density towards LMC X-1, isobaric X-ray nebular models might be more applicable for the situation prevailing in N159F. Here we note only that, according to ref. 22,

the assumption of constant pressure enhances the tendency to thermal instability in the cloud and increases the temperature and ionization gradients. The model spectra predict enhanced forbidden and semi-forbidden line intensities and relative suppression of optically allowed emission lines of highly charged ions, compared with the results obtained under the assumption of constant density.

Discussion

X-ray and ultraviolet studies of several massive X-ray binaries have revealed that these systems are imbedded in strong stellar winds (with typical mass loss rates of 10^{-7} – $10^{-6} M_{\odot} \text{ yr}^{-1}$, where $M_{\odot}$ is the mass of the Sun) emerging from the OB-type companions, which in turn will absorb most of the soft X-ray flux and cause variable P Cyg profiles of ultraviolet resonance lines formed in the wind^{32,33}. However, when the X-ray luminosity exceeds a critical value, the low- Z (atomic number) atoms which dominate the opacity of the wind will be nearly completely ionized. This situation probably prevails in the super-Eddington pulsar SMC X-1, where no indication for wind absorption has been found down to at least 0.5 keV (ref. 34). Following ref. 32 and assuming for simplicity a constant-velocity stellar wind, we find that the ionization parameter in the wind can be expressed as

$$\xi = 1.1 \times 10^5 \left(\frac{L_X}{2 \times 10^{38} \text{ erg s}^{-1}} \right) \left(\frac{a}{2 \times 10^{12} \text{ cm}} \right)^{-2} \times \left(\frac{v_w}{1,500 \text{ km s}^{-1}} \right) \left(\frac{\dot{M}}{10^{-7} M_{\odot} \text{ yr}^{-1}} \right)^{-1} \left(\frac{r_*}{r_X} \right)^2 \quad (7)$$

where r_* and r_X are the distances from the O star and X-ray component, respectively, and $\dot{M}$ denotes the mass loss rate. The units of L_X ($2 \times 10^{38} \text{ erg s}^{-1}$; see Table 1), binary separation a ($2 \times 10^{12} \text{ cm}$ (ref. 6)) and stellar wind velocity v_w ($1,500 \text{ km s}^{-1}$ (ref. 8)) have been chosen to match the observed parameters of LMC X-1. Using various mass-loss indices based on the profile of the C IV 1,548–1,550-Å ultraviolet resonance doublet³⁵, which has an absorption equivalent width of 2–4 Å (ref. 8), $10^{-7} M_{\odot} \text{ yr}^{-1}$ can probably be considered as an upper limit to the mass loss rate from star 32.

Such high ξ values are more than sufficient to completely ionize H and He ($\log \xi > 1.4$), oxygen ($\log \xi > 2$) and other low- Z atoms²¹ throughout the wind zone, with the possible exception of the X-ray shadow cast by the O star. Thus, the stellar wind in the LMC X-1 system is largely transparent to soft-X-ray and EUV photons, consistent with our observation that N159F is exposed to most of the EUV photons from an unabsorbed thermal bremsstrahlung or 'cool disk' spectrum.

We have shown that LMC X-1 is not just projected onto N159F but that the source is located within, and interacting with, the extreme population I environment. This has the important consequence that the interstellar medium around the X-ray binary is dense enough ($N_e \approx 40 \text{ cm}^{-3}$, Table 2) to reveal observable high excitation features. Keeping in mind that the extent and luminosity of the $\lambda 4,686$ emission scale with $(L_X/n)^{1/2}$ and L_X , respectively, we find that the surface brightness (intensity), $I_{4,686}$, in this approximation is directly proportional to the gas density; that is, $I_{4,686} \propto N_e$, independent of L_X . Let us imagine an LMC-X-1-type source in a low-density environment, with $N = 0.1 \text{ cm}^{-3}$ being typical for the general interstellar medium. The He III zone would then be 20 times more extended, emitting a $\lambda 4,686$ intensity of $4 \times 10^{-18} \text{ erg s}^{-1} \text{ cm}^{-2} (\text{arc s})^{-2}$ (assuming negligible reddening), which comes close to the limit of detectability for today's instrumentation.

How can we explain the fact that, with the notable exception of LMC X-1, massive X-ray binaries are generally found far away from their birthplaces? According to evolutionary scenarios for MXRB (ref. 36, and references quoted therein)

the supernova explosion creating the compact component introduces an appreciable orbital eccentricity and systemic run-away velocity, v^{ra} . When the OB star is sufficiently evolved (evolutionary timescale τ^{ev}) either to emit a strong stellar wind or fill its critical lobe, the system will enter the relatively short ($< 10^5 \text{ yr}$) X-ray phase.

For system parameters representing typical MXRB, $v^{\text{ra}} = 40$ – 100 km s^{-1} and $\tau^{\text{ev}} = 3 \times 10^6 \text{ yr}$, runaway distances of $d_{\text{ra}} = 200 v_{70}^{\text{ra}} \tau_{6.5}^{\text{ev}} \text{ pc}$ (where $v_{70}^{\text{ra}} = v^{\text{ra}}/70 \text{ km s}^{-1}$ and $\tau_{6.5}^{\text{ev}} = \tau^{\text{ev}}/10^{6.5} \text{ yr}$) from the site of formation are envisaged, displacing the X-ray binary far outside the average parent OB association and, thus far from the region of high interstellar density.

The fact that LMC X-1 is located within N159 (diameter 50 pc) argues for an exceedingly small v^{ra} ($\approx 20 \text{ km s}^{-1}$), which is consistent with the upper limit of 40 km s^{-1} (ref. 6) to the systemic relative radial velocity with respect to the nebular emission. We cannot exclude with certainty, however, that LMC X-1 was born in one of the nearby OB associations (LH103, LH108) and is by chance now crossing N159F.

We note that the BH candidate system LMC X-3 should have had a different evolutionary history. In the framework of a recently proposed evolutionary scenario for this source³⁷, we find a runaway distance $d_{\text{ra}} = 1.8 \text{ kpc}$ ($v_{70}^{\text{ra}} = 2$, $\tau_{6.5}^{\text{ev}} = 3.8$), which explains its location in the outskirts of the LMC more than 2° (corresponding to 2 kpc) away from the nearest region of recent star formation.

A possibly related high-excitation region has recently been discovered around the SNR 1E0102.2-7219 in the Small Magellanic Cloud, where a He II $\lambda 4,686$ -emitting halo surrounds the X-ray and optical remnant³⁸. The X-ray emission and its extrapolation towards lower energies, however, seems to be too weak to account for the ionization. Instead, a fossil H II region created by an ultraviolet flash during the supernova explosion or ultraviolet radiation from the SNR itself were proposed to be responsible for the excitation of the observed He III region.

Yet another candidate X-ray ionized nebula in the Magellanic Clouds was identified in the course of the present study, in the form of a shell-like structure in the light of [O III] centred on the ultra-soft LMC X-ray binary LHG83 (ref. 39). The question of whether the environment of the Magellanic Clouds is particularly favourable for the formation of observable X-ray-ionized nebulae (as seems to be the case for the formation of black holes⁴⁰), or whether some selection effect operates against their detection in the Galaxy, remains to be investigated. Further studies of galactic X-ray binaries at a sensitivity equal to or exceeding that of our observations will be necessary to search for galactic counterparts to N159F.

Conclusions

Our spectroscopic observations of LMC X-1 reveal that the black-hole-candidate binary is surrounded by a spatially resolved He III region, N159F, of $\sim 6 \text{ pc}$ in diameter. Within N159F the degree of ionization and the temperature strongly increase towards the X-ray source. We suggest that we are seeing the first example of an X-ray-ionized nebula, which is furthermore embedded in a more conventional H II region excited by the O7 star optical counterpart of LMC X-1 (star 32). Using simple photon-counting computations we have determined the previously unobservable EUV/soft-X-ray spectrum of an accreting compact object from the flux in the nebular He II $\lambda 4,686$ recombination line. Our results suggest that N159F is subjected to an essentially unabsorbed thermal bremsstrahlung (or related) X-ray spectrum, extending towards low energies to at least 0.054 keV. We show that, in contrast to the situation encountered in most galactic massive X-ray binaries, the X-ray luminosity of LMC X-1 is sufficiently high to nearly completely ionize the stellar wind from the early-type companion, which in turn becomes essentially transparent to EUV/soft X-ray photons. Evolutionary models have been shown to explain why strong

X-ray binaries are generally located outside regions of enhanced interstellar gas density, implying that observable N159F-type X-ray-ionized nebula are quite rare phenomena.

X-ray photoionization is also thought to play a major role in the formation of emission lines in active galactic nuclei (see refs 41, 42 and referenced therein), and several models of X-ray-ionized nebulae have been tailored to account for the observed continua and emission lines in these objects. In this context the highly ionized nebula around LMC X-1 might be considered to be a scaled-down version of the narrow-emission-line region in Seyfert-2- and 1.5-type galaxies. Most importantly, the combination of high X-ray luminosity and moderately low ambient gas density realized for LMC X-1 allows a spatially resolved study

of the ionization, temperature and density structure, whereas active galactic nuclei appear largely unresolved in optical and ultraviolet observations.

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Note added in proof: Our recent detection in extended [Ne V] $\lambda 3426$ emission around LMC X-1 with ionization potential (to produce the ion) of 97.1 eV provides further support to the X-ray-photoionization model of N159F.

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2.5-Myr *Australopithecus boisei* from west of Lake Turkana, Kenya

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Specimens of Australopithecus boisei have been found in 2.5-Myr-old sediments west of Lake Turkana, Kenya. The primitive morphology of these early A. boisei suggests that robust and hyper-robust Australopithecus developed many of their common features in parallel and further that A. africanus is unlikely to have been ancestral to A. boisei.

THE 'hyper-robust' hominid *Australopithecus boisei* is well-known from several East African Plio-Pleistocene deposits dated between 2.2 and 1.2 Myr (refs 1, 2). It has been thought of variously, as: the northern vicar of the equally well-known *A. robustus*³; the extremely specialized end-member of the robust clade⁴; an already developed species which immigrated from another, unknown area⁵; and as representing individuals at the large end of a single *Australopithecus* species that also encompasses *A. robustus* and *A. africanus*⁶.

There is a growing consensus that the east and south African samples are different enough to allow them to be placed in separate species^{1–4}. The type specimen of *A. boisei* is Olduvai Hominid 5^{7,8}.

These two robust species are placed in the genus *Paranthropus* by some authors⁹. Although recognizing that the two known samples overlap in time, some have advocated an ancestor-descendant relationship with *A. robustus* giving rise to *A. boisei*. Perhaps the most compelling recent evidence for this last view

is Rak's exemplary study of the structure and function of the australopithecine face⁴. He has followed an evolutionary scheme in which the origins of the robust clade are in *A. africanus*, which is thereby removed from consideration as a human ancestor¹⁰. This scheme has not found universal acceptance^{11,12}.

Localities

Prospecting was carried out in 1985 in Pliocene sediments to the west of Lake Turkana, Kenya. It led to the recovery of two *A. boisei* specimens. A cranium and a partial mandible were discovered at two separate localities: sediments in the Lomekwi and Kangatukuseo drainages (see Fig. 1) at approximately 3°45' N, 35°45' E.

The general dip of strata is to the east in the Lomekwi drainage, but the strata at Lomekwi I from which the cranium was derived are deformed into a syncline by drag along a fault that truncates the section about 50 m east of the site. Several small faults cut the section west of the site, but it has been possible to link the

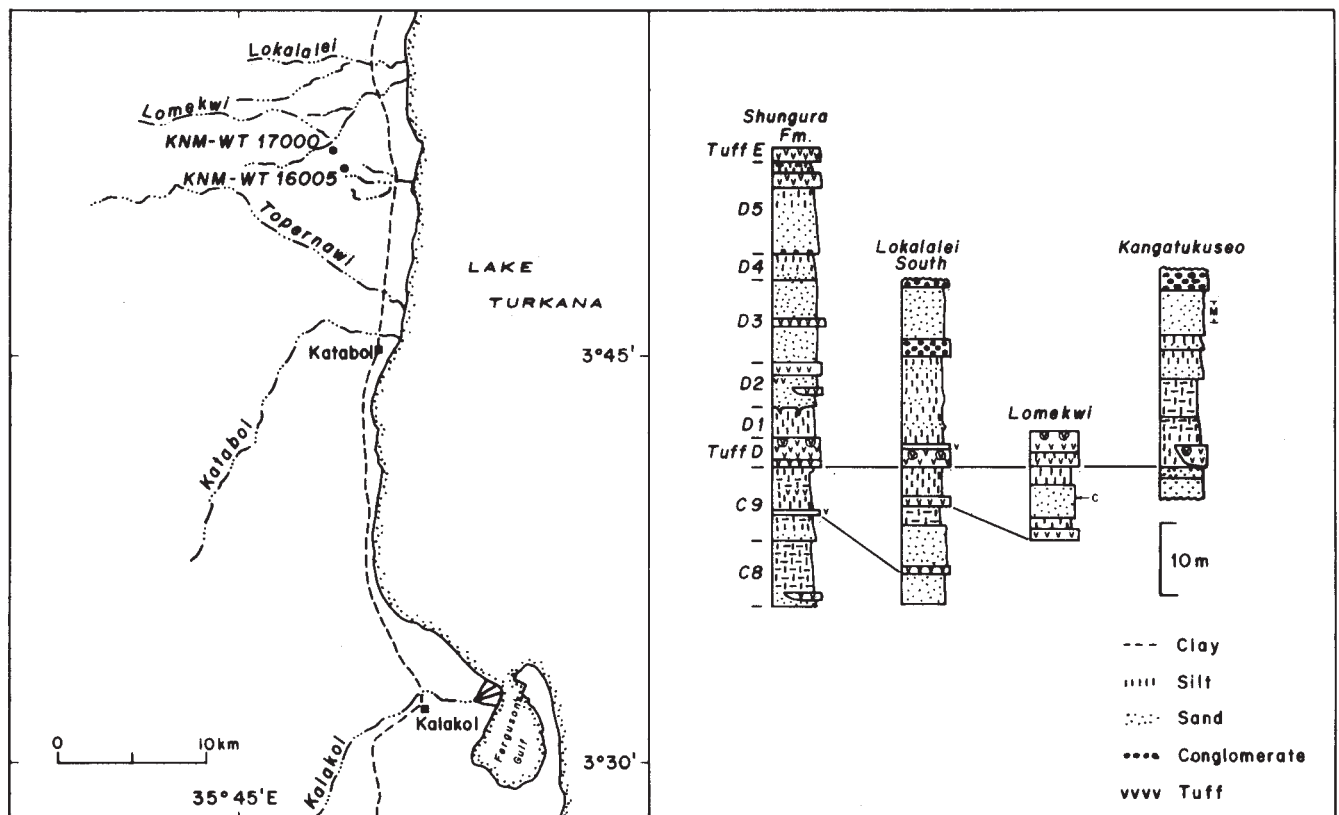


Fig. 1 Map and sections showing geographical and stratigraphic positions of KNM-WT 17000 and KNM-WT 16005. C, cranium; M, mandible.

short sections together by analysis of volcanic ash layers in the sequence. The total thickness of section immediately surrounding the site is less than 15 m, and the volcanic ash layer which caps the section is compositionally indistinguishable from Tuff D of the Shungura Formation. Earlier¹³, this tuff was referred to the informally designated Upper Burgi Tuff of the Koobi Fora Formation. With more numerous analyses it is now clear that the Upper Burgi Tuff also correlates with Tuff D, and an additional correlation datum is provided between the Shungura Formation and the Koobi Fora Formation. Tuff D has been

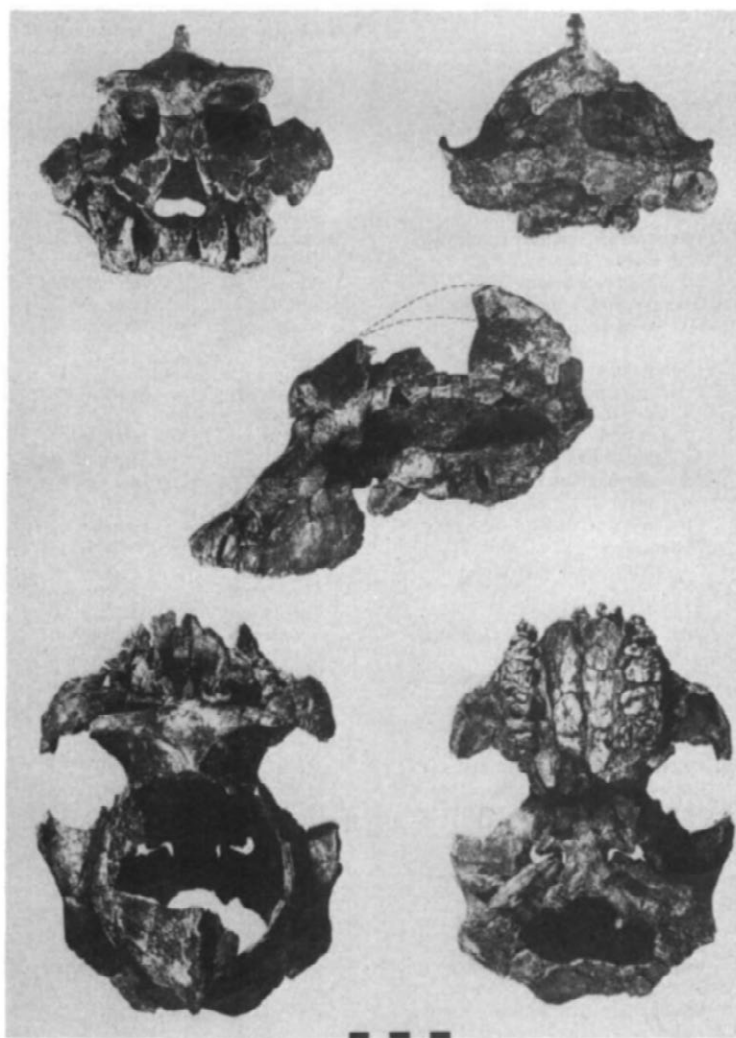
dated at 2.52 ± 0.05 Myr¹⁴. This age is an average computed from samples from the Shungura Formation in Ethiopia, and from the correlative unit at Kanganatukuseo. The cranium derives from a level 3.8 m below Tuff D. Around 10 m below Tuff D there is a second ash layer. In the Lokalelei drainage, about 4 km north-west of the site, there are three ash layers exposed in sequence. The lowest correlates with a tuff in submember C9 of the Shungura Formation, and the upper two correlate with the two ash layers exposed at the site of the cranium. On this basis, the cranium is shown to lie within strata correlative with submember C9 of the Shungura Formation. Based on the K/Ar chronology of the Shungura Formation and scaling on the basis of constant sedimentation rates there, the cranium is estimated to be 2.55 Myr old. Using palaeomagnetic polarity boundaries as the basis of chronological placement, the age of the cranium would be ~ 2.45 Myr, as there is a slight discordance between the two chronologies¹⁵. Therefore, we believe that the age of the cranium can be confidently stated as 2.50 ± 0.07 Myr, including all errors. The sediments from which the cranium derives are overbank deposits of a large perennial river, probably the ancestral Omo.

The mandible from Kanganatukuseo III derives from a level about 19 m above Tuff D. Tuff E is not exposed in Kanganatukuseo, but it is exposed in the northern part of the Lomekwi drainage, and in the southern Lokalelei drainage. There, sediments correlative with Member D of the Shungura Formation are at least 26 m thick. On this basis, the mandible is assigned to the central part of Member D of the Shungura Formation, the best age estimate for which is 2.45 ± 0.05 Myr. The section is faulted east of the mandible site, and older sediments are exposed along the drainage east of that point. In fact, the entire section exposed in Kanganatukuseo lies within an interval from ~ 25 m above Tuff D to 5 m below that tuff. The mandible was collected from a sandstone layer ~ 6 m thick, deposited by a large river system. Thin basalt pebble conglomerates intercalated in this part of the section show that the site lay near the boundary between sediments deposited by this large river, and alluvial fan deposits derived from the west.

Table 1 Fossil mammals from the new australopithecine localities west of Lake Turkana

<i>Theropithecus brumpti</i>	<i>Giraffa</i> sp.
<i>T. cf. brumpti</i>	<i>Aepyceros</i> sp.
Cercopithecidae large	<i>Connochaetes</i> sp.
Cercopithecidae medium	<i>Parmularius cf. braini</i>
Cercopithecidae small	Alcelaphini medium
Papionini medium/large	Alcelaphini small
<i>Parapapio ado</i>	<i>Menelikia</i> sp.
<i>Paracolobus mutiwa</i>	<i>Kobus sigmoidalis</i>
<i>Australopithecus boisei</i>	<i>Kobus</i> sp. A
<i>Hyaena</i> sp.	<i>Kobus</i> sp. B
<i>Homotherium</i> sp.	<i>Kobus</i> sp. C
Felidae	<i>Kobus</i> sp. D
Viverridae	<i>Kobus</i> sp. E
Carnivora indet.	<i>Kobus</i> sp. F
<i>Deinotherium bozasi</i>	Reduncini indet.
<i>Elephas recki shungurensis</i>	Reduncini large
<i>Hipparion hasumensis</i>	Reduncini medium
<i>Ceratotherium</i> sp.	Reduncini small
<i>Diceros bicornis</i>	<i>Tragelaphus nakuae</i>
<i>Notochoerus scotti</i>	<i>Tragelaphus</i> sp.
<i>Kolpochoerus limnetes</i>	Bovini
<i>Hexaprotodon protamphibius</i>	<i>Gazella aff. granti</i>
<i>Hippopotamus imagunculus</i>	<i>Antidorcas recki</i>
<i>Camelus</i> sp.	Antilopini
<i>Sivatherium maurusium</i>	

Fig. 2 Anterior, posterior, left lateral, superior and inferior views of cranium KNM-WT 17000. Scale in cm.



The interval of section from which the australopithecine specimens were collected has also yielded over 200 fossils representing more than 40 other mammalian species (Table 1), including the skeleton of a ground-dwelling colobine and a relatively complete camel mandible. Bovids are the most common elements of the fauna at this level and most represent species that are otherwise known from the lower portion of the Omo Shungura succession. But whereas alcelaphines and impalas predominate at lower horizons west of Lake Turkana, reduncine bovids are the commonest fossils at the australopithecine sites reported here and are taxonomically different from those recovered slightly higher in the sequence. *Elephas recki shungurensis* is the common elephant at the localities considered here while *Notochoerus scotti* and *Kolpochoerus limnetes* are the common suids. Although all three taxa have lengthy Pliocene distributions, the West Turkana specimens closely match samples of these species from Omo Shungura Members C and D—thus supporting the estimate of age derived from the tuff analyses. The age estimate is corroborated by the apparent absence of the elephantids *Loxodonta adaurora*, *Loxodonta exoptata* and *Elephas recki brumpti*, and of the suids *Nyanzachoerus kanamensis*, *Notochoerus euilus*, *Kolpochoerus afarensis* and *Potamochoerus* sp., all of which occur in older horizons in the upper reaches of the Laga Lomekwi. Also missing from the australopithecine-bearing assemblage are the reduncine bovid

Menelikia lyrocera and equids of the genus *Equus*, both of which occur higher in the sequence.

Specimens

KNM-WT 17000 is an adult cranium with the following parts missing: all of the tooth crowns except a half molar and the right P^3 ; some facial bone fragments which have spalled off the infilled maxillary sinus; most of the frontal processes and temporal plates of the zygomatics; the zygomatic arches themselves; a large part of the frontal and parietals superiorly (but a piece of the sagittal crest on the anterior part of the parietal is preserved); parts of both pterygoid regions inferiorly and the posterior part of the maxilla and palate on the right side; and the inferior part of the nuchal region of the occipital. There is no bilateral asymmetry and all bony contacts are sharp. There is no evidence of any plastic deformation and the brain case has retained its spheroidal shape (Fig. 2).

It is a massively-built cranium with a very large facial skeleton, palate and large cranial base, but with a small brain case. The palate and cranial base are roughly the same size as in Olduvai Hominid (O.H.) 5. The cranial base is about the same size as, but the palate is slightly larger than in, KNM-ER 406 (ref. 16). The cranial capacity is 410 ml (mean of five determinations by water displacement with a standard error of 4.32). This measurement is probably accurate since the orbital plates of the frontals,

Table 2 Compilation of features of *Australopithecus* species

Feature	<i>A. afarensis</i>	<i>A. africanus</i>	<i>A. robustus</i>	<i>A. boisei</i>	KNM-WT 17000
Position of I ² roots relative to nasal aperture margins	Lateral	Medial	Medial	Medial	Medial
Divergence of temporal lines relative to lambda	Below	Above	Above	Above	Below
Lateral concavity of nuchal plane	Present	Absent	Absent	Absent	Probably present
Depth of mandibular fossa	Shallow	Deep	Deep	Deep	Shallow
Temporal squama pneumatization	Extensive	Weak	Weak	Weak	Extensive
Flat, shallow palate	Present	Absent	Absent	Absent	Present
Subnasal prognathism	Pronounced	Intermediate	Reduced	Reduced	Pronounced
Orientation of tympanic plate	Less vertical	Intermediate	Vertical	Vertical	Intermediate
Flexion of cranial base	Weak	Moderate	Strong	Strong	Weak
Relative sizes of posterior to anterior temporals	Large	Intermediate	Small	Small	Large
Position of postglenoid process relative to tympanic	Completely anterior	Variable	Merge superiorly	Merge superiorly	Completely anterior
Tubular tympanic	Present	Intermediate	No	No	Intermediate
Articular eminence	Weak	Intermediate	Strong	Strong	Weak
Foramen magnum relative to tympanic tips	Anterior	Intermediate	Anterior	Anterior	Anterior
Coronally placed petrous temporals	No	Variable	Yes	Yes	Yes
Distance between M ¹ and temporomandibular joint	Long	Variable	Short	Short	Long
P ³ outline	Asymmetric	Intermediate	Oval	Oval	Oval
Relative size of C	Very large	Medium	Small	Small	Small
Anterior projection of zygomatic	Absent	Intermediate	Strong	Very strong	Very strong
Height of masseter origin	Lowest	Intermediate	High	High	High
Canine jugum separate from margin of pyriform aperture	Yes	Variable	No	No	No
Distinct subnasal and intranasal parts of clivus	Yes	Intermediate	No	No	No
Relative size of post-canine teeth	Moderate	Large	Very large	Very large	Very large
Robustness of zygomatic arches	Moderate	Strong	Very strong	Very strong	Very strong
Common origin of zygomatic arch	M ¹ /P ⁴	P ⁴	P ⁴	P ³	P ³
C jugum	Prominent	Pronounced	Reduced	Lost	Lost
Inclination of nuchal plane	Steep	Less steep	—	Variable	Less steep
Compound temporonuchal crest	Present	Absent	Males only	Males only	Present
Asterionic notch	Present	Absent	Absent	Absent	Probably present
Medial inflection of mastoids	Strong	Reduced	Reduced	Reduced	Reduced
Anterior facial pillars	Absent	Present	Present	Absent	Absent
Length of nuchal plane relative to occipital	Long	Intermediate	Long	Long	Long
Braincase relative to face	—	High	Low	Low	Low
Nasals wide above frontonasal suture	—	No	Yes	Yes	Yes
Nasoalveolar gutter present	No	No	Yes	Yes	Yes
Infraorbital foramen high	Yes	Yes	No	No	No
Maxillary fossula present	No	Yes	Yes	No	No
Inferior orbital margins soft laterally	—	No	Yes	No	Yes
Greatest orbital height	—	Middle	Middle	Medial	Middle
Foramen magnum heart-shaped	—	No	No	Yes	Yes

the cribiform plate region of the ethmoid, one anterior clinoid and both posterior clinoid processes are preserved together with the rest of the cranial base. The missing cranial vault fragments can be reconstructed with fair certainty by following the internal contours all around them. This is the smallest published cranial capacity for any adult fossil hominid, although A.L. 162-28 from Hadar¹⁷ must have been smaller. Given the massive face and palate combined with a small brain case, it is not surprising that the sagittal crest is the largest ever in a hominid. Further, the sagittal crest joins completely to compound temporal-nuchal crests with no intervening bare area¹⁸. The foramen magnum position is far forward as in other robust *Australopithecus* specimens¹⁹.

The one complete tooth crown, right P³, is 11.5 mesiodistally by 16.2 buccolingually. This is bigger mesiodistally (md) than O.H. 5 (10.9) and smaller buccolingually (bl) (17.0)^{7,8}. These dimensions are completely outside the recorded range for *A. robustus* (9.2–10.7 md, 11.6–15.2 bl) and at the high end of the range for *A. boisei* (9.5–11.8 md, 13.8–17.0 bl)²⁰. Only the largest *A. boisei* mandibles found so far (for example, KNM-ER 729

and 3230²¹) would fit this cranium. It is unfortunate that the region of the occipital which would show the grooves for the occipital and marginal sinuses is missing, but the small sigmoid sinuses appear to have no contribution from transverse ones. Thus we feel that enlarged occipital and a marginal sinuses may have been present.

Most of the previously recorded differences between *A. boisei* and *A. robustus* have involved greater robustness in the former. In fact for those parts preserved in KNM-WT 17000, the definitions originally given by Tobias⁸ include only two characters that cannot be simply attributed to this robustness. One is that the supraorbital torus is 'twisted' along its length. Subsequent discoveries of *A. boisei* specimens show that O.H. 5 is extreme in its supraorbital torus development and that others are not so 'twisted'. The other character is that *A. boisei* palates are deeper anteriorly than those of *A. robustus*, in which they tend to be shallow all along the length. Recently, Rak⁴ has undertaken a study of the australopithecine face and has documented structural differences between the faces of all four species. Skelton *et al.*²² have just made a cladistic analysis of



Fig. 3 Occlusal view of mandible KNM-WT 16005. Scale in cm.

early hominids; Table 2 lists some of the characteristics given as typical of *Australopithecus* species by these authors (see ref. 22 and refs therein) as well as the condition found in KNM-WT 17000. For most features the new specimen resembles *A. boisei*.

There are some features of KNM-WT 17000 that differ from all other 'hyper-robust' specimens as well as from robust ones. The most obvious and important is the prognathic mid- and lower facial region. In superior view all other robust crania are so orthognathic that only a small part of the incisor region projects past the supraorbital tori. In KNM-WT 17000 the mid-face projects strongly past the tori and the anterior maxilla projects well forwards as a square muzzle. In summary, we regard this specimen as part of the *A. boisei* clade and view its differences from the younger sample as being either primitive, or part of normal intraspecific variation that has not been documented before, or both.

Mandible KNM-WT 16005 has the body preserved to the M_3 alveoli on the left and the M_2 alveoli on the right. The base is missing. The incisors and canines are, as judged from their roots, relatively very small and the post-canine teeth relatively very large. In its size, shape and proportions, KNM-WT 16005 is very similar to the Peninj mandible²³, except that the P_4 and M_1 of the latter are a little larger and the M_2 a little smaller than this specimen. KNM-WT 16005 is smaller than the mandible which KNM-WT 17000 possessed. Tooth measurements are given in Table 3, and the specimen is shown in Fig. 3.

Although future finds may show that KNM-WT 17000 is well within the range of variation of *A. boisei*, it is also possible that the differences will prove sufficient to warrant specific distinction. If the latter proves to be the case we suggest that some specimens from the same time period and from the same sedimentary basin (for example, Omo 1967-18 from the Shungura Formation) will be included in the same species. Omo 1967-18 is the type specimen of *Paraustralopithecus aethiopicus* Arambourg and Coppens²⁴. In our view, the appropriate name then would be *Australopithecus aethiopicus*.

Conclusions

The new specimens show that the *A. boisei* lineage was established at least 2.5 Myr ago and further that, in robustness and tooth size, at least some members of the early population were as large as any later ones. Although one authority suggested

that the robust australopithecines became smaller in skull and tooth size with time²⁵, most have pointed out that the available sample showed the opposite, that within *A. boisei* there has been an increase in size and robustness of the skull and jaws. This was apparently an artefact of sampling and is no longer correct.

Although recognizing that at least some populations of *A. robustus* and *A. boisei* overlapped in their time ranges, Rak⁴ hypothesized that the former was ancestral to *A. boisei*. This is no longer tenable. *A. robustus* shares with younger examples of *A. boisei* several features which are clearly derived from the condition seen in KNM-WT 17000. These include the cresting pattern—with the emphasis on the anterior and middle parts of the temporalis muscle—the orthognathism and the deep temporomandibular joint with strong eminence. At the same time KNM-WT 17000 is clearly a member of the *A. boisei* lineage, as demonstrated by the massive size, extremely large palate and teeth, the build of the infraorbital and nasal areas and the anterior position and low take-off of the zygomatic root.

Therefore, this new specimen shows that *A. robustus* is a related, smaller species that was either derived from ancestral forms earlier than 2.5 Myr and/or has evolved independently in southern Africa, perhaps from *A. africanus*. It has been suggested before that *A. robustus* was derived from *A. africanus*⁴, but by those who believed *A. robustus* then gave rise to *A. boisei*—an interpretation that is now unlikely.

The idea that *A. africanus* was the earliest species of a lineage in which *A. robustus* led to *A. boisei* is challenged by the new evidence. KNM-WT 17000 shows that all known *A. africanus* share features which are derived relative to it. Many of these same features were cited by White *et al.*²⁰ in arguing that *A. afarensis* is more primitive than *A. africanus*. Features showing KNM-WT 17000 to be more primitive than *A. africanus* that were also used to distinguish the primitiveness of *A. afarensis*

Table 3 Tooth measurements of KNM-WT 16005 (mm)

	Mesiodistal	Buccolingual
Left P_3	10.7	13.8
P_4	(12.0)	(15.0)
M_1	15.7	14.3
M_2	(17.0)	16.7

are: a very flat, shallow palate; pronounced subnasal prognathism; compound temporal/nuchal crests; sagittal crest with emphasis on posterior fibres of the temporalis muscle; an extensively pneumatized squamous temporal, which in KNM-WT 17000 is 11.5 thick just above the supraglenoid gutter; small occipital relative to nuchal plane; pneumatization of lateral cranial base to produce strongly flared parietal mastoid angles; shallow and mediolaterally broad mandibular fossae; tympanics completely posterior to the postglenoid process. In KNM-WT 17000, the asterionic region is poorly preserved, but an asterionic notch was probably present, which is an additional feature also cited to demonstrate the primitiveness of *A. afarensis*.

Other primitive features found in KNM-WT 17000, but not known or much discussed for *A. afarensis*, are: very small cranial capacity; low posterior profile of the calvaria; nasals extended far above the frontomaxillary suture and well onto an uninflated glabella; low calvaria with receding frontal squama; and extremely convex inferolateral margins of the orbits such as found in some gorillas. Thus there are many features in which KNM-WT 17000 is more primitive than *A. africanus* and similar to *A. afarensis* yet KNM-WT 17000 is clearly a member of the *A. boisei* clade. Further, although the dating of the South African sites is admittedly still imprecise and populations of ancestral species may survive a speciation event, the time sequence of the fossils is becoming increasingly less supportive of the idea of an *africanus-robustus-boisei* lineage.

Finally, it is striking that many of the features of this cranium shared by *A. afarensis* are primitive and not found in *A. robustus* or later specimens of *A. boisei*. These primitive features shared by KNM-WT 17000 and *A. afarensis* are almost exclusively confined to the calvaria, despite the largely complete face of KNM-WT 17000 and the existence of several partial facial specimens at Hadar. However, not one individual adult specimen of *A. afarensis* preserves a facial skeleton attached to a calvaria. This observation raises two alternatives: first, that these features are primitive to the Hominidae and therefore not of great taxonomic value in determining relationships among hominids; second, that, as Olson²⁶ has suggested, the specimens identified as

A. afarensis include two species, one of which gives rise directly to *A. boisei*. Whatever the final answer, these new specimens suggest that early hominid phylogeny has not yet been finally established and that it will prove to be more complex than has been stated.

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LETTERS TO NATURE

Steep-spectrum radio lobes near the galactic centre

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In a previous paper¹ we reported the discovery of an extended steep-spectrum radio lobe located ~34 arc min north-east of the galactic centre (the northern galactic centre lobe). Based on the similarity of the observed properties of this object to the radio lobes observed in the nuclei of Seyfert galaxies², we suggested that this source may be a manifestation of nuclear activity associated with the compact non-thermal source³ located at the galactic centre. However, no clear evidence for a second opposing lobe was evident from the 80-MHz observations. Here we report observations at 110.6 and 123.0 MHz which confirm the existence of a second steep-spectrum feature, first discovered by Yusef-Zadeh *et al.*⁴ at 160 MHz, which extends ~10 arc min to the south-east of the radio source Sagittarius-A. These new observations are in the only frequency range in which it is possible to view both of these features simultaneously and to display their morphology as a function of frequency. At lower frequencies the features are obscured by thermal absorption; at higher frequencies they blend into the background emission. Based on this unique perspective, we propose that these radio sources are related, and associated with activity at the galactic nucleus.

Our observations were made in April-May 1985 with the TPT telescope of the Clark Lake Radio Observatory⁵. This instrument is a T-shaped array of 720 conical-spiral antennas, measuring 3.0×1.8 km, operating in the range 15-125 MHz. The beam-width in the direction of the galactic centre is 3.6×6.2 arc min at 110.6 MHz and 3.3×5.8 arc min at 123.0 MHz. The 110.6-MHz map is the sum of three nights' observations; two nights' observa-

Table 1 Observed properties of features located near the galactic centre

	110.6-MHz flux density (Jy)	Luminosity (erg s ⁻¹)	α
Northern lobe	65	2×10^{34}	≤ -1.0
Jet feature	10	2×10^{33}	≤ -0.7
Central compact source	—	2×10^{34}	≈ -0.25

tions are summed at 123.0 MHz. Maps made from each night individually and maps made from data taken before and after transit are all consistent with each other. Observations of sources from the 3C and Culgoora catalogues were used for flux calibration and indicated that ionospheric refraction was not important. Flux densities have been referred to the absolute scale of Baars *et al.*⁶.

Figure 1 shows a superposition of the relevant portions of the Clark Lake 110.6- and 123.0-MHz maps on the 160-MHz Culgoora radiotelescope map of Yusef-Zadeh *et al.*⁴. The peak of the 160-MHz emission is located at (RA = 17 h 42 min 36.7 s, dec. = -28° 58' 17"), which is coincident with the northeastern part of the non-thermal Sgr-A radio shell. The emission extending 10 arc min to the south-east of Sgr-A has been identified as a low-energy jet and is hereafter referred to as the jet feature. Our 123.0-MHz map is in excellent agreement with the Culgoora map and clearly confirms the existence of extended steep-spectrum emission in this region. However, both maps are still dominated by emission from Sgr-A.

At 110.6 MHz the emission from Sgr-A has weakened considerably relative to the emission from the steep-spectrum jet feature lying to the south-east. This is clearly illustrated by the portion of the 110.6-MHz map shown in Fig. 1. This result is partly due to the steep spectrum of the jet feature, and also to the turnover in the spectrum of Sgr-A. (Sgr-A is surrounded by a halo of ionized gas^{7,8} which causes absorption at low frequencies.)

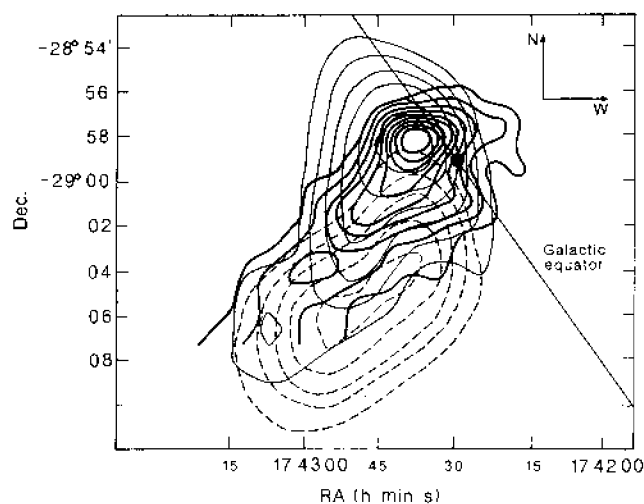


Fig. 1 Contour maps of the galactic centre jet feature. Thick lines, 160-MHz Culgoora map: beam-width, 1.9×1.9 arc min; first contour, 1.2 Jy per beam area; contour interval, 1.2 Jy per beam area. Thin lines, 123-MHz Clark Lake map: beam-width, 3.3×5.8 arc min; peak brightness temperature, 49,193 K; first contour, 19,120 K; contour interval, 5,461 K. Dashed lines, 110.6-MHz Clark Lake map: beam-width, 3.6×6.2 arc min; peak brightness temperature, 10,770 K; first contour, 2,700 K; contour interval, 1,795 K. The black dot indicates the position of the galactic centre ($l=0$, $b=0$).

Figures 2 and 3 show the complete 123.0- and 110.6-MHz maps. The three most prominent emission features on the 123.0-MHz map are the continuum arc (G0.16-0.15), the northern galactic centre lobe and Sgr-A. At 110.6 MHz the steep-spectrum jet feature has replaced Sgr-A as a prominent source in the field. The dashed lines on these maps correspond to regions of absorption produced by ionized gas along the line of sight¹.

The 110.6-MHz map (Fig. 3) is important because it reveals the similarities of the northern galactic centre lobe and the jet feature. Both of these objects are extended, steep-spectrum features which are seen only at low frequencies. Moreover, whereas the jet feature appears to be directly linked to the centre, the 110.6-MHz map indicates that the northern lobe extends towards Sgr-A and thus may also be physically connected to the centre. In fact, we would not expect to observe any connection at these frequencies because of the absorption caused by thermal gas in the region between the northern lobe and the centre. The absorption region observed at 110.6 and 80 MHz coincides with a peak in the 55- and 125- μ m infrared emission⁹. Unfortunately, the 160-MHz map does not extend far enough north to include the northern lobe.

Recent observations^{10,11} at 10 GHz have also revealed an apparently separate core-lobe system associated with the non-thermal portion of the continuum arc, G0.16-0.15. Evidence for these features is present on our 110.6-MHz maps ('arc lobes'); the observation of these features at low frequencies leaves no doubt as to their non-thermal nature.

Table 1 summarizes the observed properties of the northern galactic centre lobe, the jet feature and the central compact source. We have assumed standard synchrotron source theory. Accurate measurement of the spectral index (α) of the jet feature between 110.6 and 160 MHz is not possible because it depends crucially on the knowledge of the flux contribution from Sgr-A. Also, the spectrum of the jet is subject to significant free-free absorption. We have estimated an upper limit for the spectral index of the jet feature by comparison with higher-frequency maps where this object is not detected. The result of this estimate is $\alpha \leq -0.7$, consistent with the estimate by Yusef-Zadeh *et al.*⁴ of $\alpha \leq -0.85$. A new estimate of the spectral index of the northern radio lobe based on the 110.6-MHz map and higher-frequency

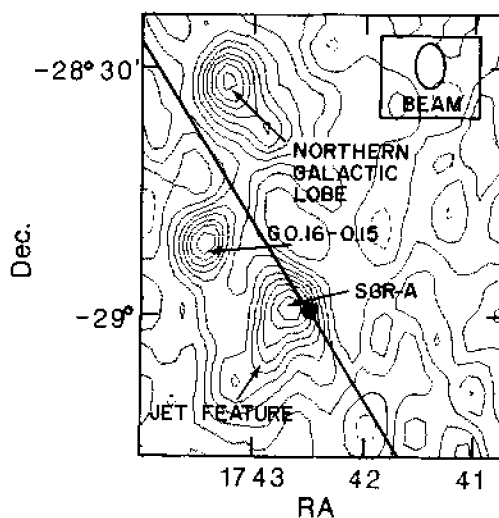


Fig. 2 Contour map at 123 MHz of the galactic centre region: beam-width, 3.3×5.8 arc min; peak brightness temperature, 54,600 K; first contour, 2,753 K; contour interval, 5,461 K. Black dot, galactic centre.

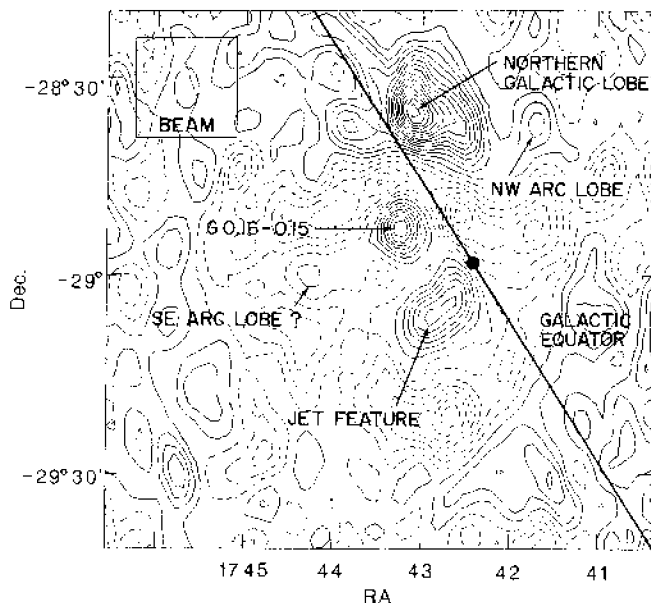


Fig. 3 Contour map at 110.6 MHz of the galactic centre region: field, $1.4^\circ \times 1.4^\circ$; beam-width, 3.6×6.2 arc min; peak brightness temperature, 26,994 K; first contour, 905 K; contour interval, 1,795 K. Black dot, galactic centre.

maps is consistent with the limit of $\alpha \leq -1.0$ obtained previously¹.

We propose that the northern galactic centre lobe and the jet feature have been produced by similar processes associated with the galactic nucleus. In their geometry and other properties, these objects bear a strong resemblance to features observed in the nuclei of Seyfert and active galaxies^{2,12}. These similarities include the spatial scale of the lobes with respect to the central compact source, the fact that the luminosity of these lobes is similar to that of the central compact source, and the fact that the spectra of the lobes are much steeper than that of the central compact source. Similar radio features have been observed in several apparently normal spiral galaxies^{13,14}, substantiating the claim that Seyfert-like activity may be common among spiral galaxies^{15,16}. Asymmetry of these features with respect to Sgr-A

is not surprising. The asymmetrical geometry of these lobes is reminiscent of the 'dog leg' structures observed in several quasars¹⁷. These 'dog leg' structures are most probably produced by a collision of the radio lobe with an intergalactic cloud. Given the lower energy of the galactic features and the density of interstellar clouds near the galactic centre, bent radio lobe structures would be probable. Our observations are evidence for activity at the galactic centre driven by physical mechanisms which are similar¹⁸, although scaled down, to those which operate in active galaxies and quasars.

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An interstellar line coincident with the P(2,1) transition of hydronium (H_3O^+)

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Gas-phase interstellar chemistry is thought to be dominated by ion-molecule reactions. Neutral molecular species abound in the dense interstellar clouds, but only seven molecular ions have been detected. Hydronium (H_3O^+) is predicted in all ion-molecule reactions¹⁻³ to be an abundant molecular ion, forming OH and H_2O by dissociative electron recombination reactions. Here we report the detection, towards the Orion-KL nebula, of a weak emission spectral line coincident in frequency with the P(2,1) rotation-inversion transition of H_3O^+ . Although we cannot be certain that this single-line detection is H_3O^+ , we evaluate this possibility with regard to its observed line parameters and, using theoretical chemical models, derive an Orion fractional abundance for H_2O (relative to H_2) of 10^{-5} and 10^{-6} in thermal and non-thermal cases, respectively. We also report 1-mm-wavelength observations towards Orion which yield new interstellar transitions of CH_3OH , $^{34}\text{SO}_2$, H_2CS , SO, OCS, SiO and two unidentified lines at 307,313 and 304,374 MHz.

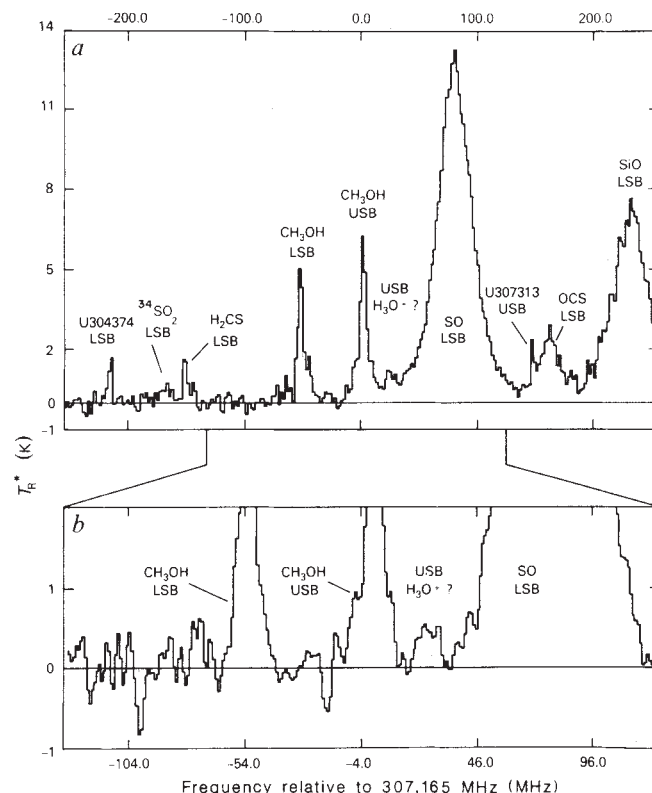


Fig. 1 *a*, Orion-KL line spectrum at 2 MHz resolution (see Table 1). The abscissa is USB frequency relative to 307,165 MHz, assuming a source V_{LSR} of +8.8 km s⁻¹. As this spectrum represents the alignment and averaging of data taken at centre frequencies of both 307,165 and 307,161 MHz, to de-correlate filters, only the USB features are displayed at the correct frequency; the LSB lines are smeared across two channel widths (4 MHz). These data represent 7.1 h of integration time. *b*, Smoothed version (Hanning smooth) of a portion of the Orion-KL line spectrum at 1 MHz resolution; abscissa as in *a*. The LSB lines are smeared across four channel widths (4 MHz). These data represent 7.3 h of integration time.

OH and H_2O are well-known and abundant constituents of interstellar clouds, but H_2O does not possess easily observable transitions from which abundances can be determined. In interstellar molecular sources, successful observations of interstellar H_3O^+ would provide an indirect method of estimating H_2O abundances⁴. Several transitions of the rotation-inversion spectrum of H_3O^+ have now been measured in the laboratory^{5,6}, making possible an interstellar search for this ion. The P(2,1) transition is at a rest frequency of 307,192.41(5) MHz, and the P(3,K) transition with $K=0, 1$ and 2 lies between 360 and 400 GHz. The upper energy level of the P(2,1) transition is ~105 K above the ground state; this relatively low degree of excitation is seen in other molecular species in molecular clouds such as Orion and SgrB2. Towards the Orion-KL nebula we detected a weak emission feature, coincident in frequency with the P(2,1) transition of H_3O^+ , with line parameters ($T_{\text{R}}^* \approx 0.6$ K, $\Delta V \approx 10$ km s⁻¹, $V_{\text{LSR}} \approx 11$ km s⁻¹), which would be consistent with a weak molecular species detected in the 'plateau' or 'doughnut' region of this source^{7,8}.

Although this feature is by no means certain to be attributable to H_3O^+ , we have no other candidate for its identification, and, because of the importance of this species to interstellar chemistry, we are reporting our preliminary observations now and expect that we or others will confirm our present work with further observations of the P(2,1) and the higher-frequency P(3,K) transitions.

Table 1 Observed line parameters

Molecule	Transition	Frequency (MHz)	Band	Orion-KL†				SgrB2‡			
				Resol. (MHz)	T_R^* (K)	ΔV (km s ⁻¹)	V_{LSR} (km s ⁻¹)	Resol. (MHz)	T_R^* (K)	ΔV (km s ⁻¹)	V_{LSR} (km s ⁻¹)
U307313§	—	307,313 (1)	USB	1	1.0 (2)	4 (1)	8.8 (10)				
H ₃ O ⁺ ?	P(2, 1)	307,192.41 (5)	USB	1	0.6 (1)	9.6 (20)	11 (1)				
CH ₃ OH	4 ₁ -4 ₀ A	307,165.94 (5)	USB	1	6.6 (1)	6 (1)	8.8 (10)	2	0.8 (1)	17 (2)	53 (2)
U304374	—	304,374 (1)	LSB	2	1.6 (2)	5 (2)	8 (2)				
³⁴ SO ₂	3 _{3,1} -2 _{2,0}	304,332.1 (2)	LSB	2	0.7 (2)	29 (2)	8 (2)				
H ₂ CS	9 _{1,9} -8 _{1,8}	304,305.97 (50)	LSB	2	2.0 (2)	5 (2)	8 (2)				
CH ₃ OH	2 ₁ -2 ₀ A	304,208.35 (5)	LSB	1	7.2 (3)	5 (1)	8 (1)	2	0.5 (1)	14 (2)	51 (2)
³² SO	8 ₇ -7 ₆	304,077.84 (10)	LSB	2	13 (1)	36 (2)	6 (1)	2	0.6 (1)	20 (4)	62 (2)
OCS	25-24	303,993.24 (10)	LSB	2	3.3 (10)	7.8 (20)	5 (2)				
SiO $v=0$	7-6	303,926.96 (10)	LSB	2	8 (1)	32 (4)	6 (2)				

Line parameters T_R^* , antenna temperature; ΔV , line-width; V_{LSR} , velocity with respect to the local standard of rest.

† Orion-KL J1950.0 position: (05 h 32 min 47 s, -05° 24' 21").

‡ SgrB2 J1950.0 position: (17 h 44 min 11 s, -28° 22' 30").

§ The 4₁-4₀ A transition of ¹³CH₃OH is calculated to be 307,311.4 (20) MHz; laboratory measurements are pending.

The double-sideband observations were made on 12–16 March 1986 with the National Radio Astronomy Observatory (NRAO) 12-m radio telescope. The single-channel, cooled mixer receiver has a single-sideband receiver temperature of ~4,000 K. The sideband separation was 3,000 MHz with a spectrometer consisting of two 256-channel filter banks with 1-MHz and 2-MHz filters. The upper sideband (USB) contained the H₃O⁺ line frequency. Calibration by the chopper method was used to correct for telescope losses and atmospheric extinction. Atmospheric conditions were highly variable, although some stable conditions did occur during observations of Orion and SgrB2. During the stable periods, zenith opacities for Orion observations on 12, 14 and 15 March were 1.1, 1.1 and 0.3, respectively; for SgrB2 on 13, 15 and 16 March they were 0.5, 0.3 and 0.2, respectively. The half-power beam-width was ~21 arc s at 307 GHz. Data were taken while the beam position was switched by 1° in azimuth for SgrB2 and 0.5° for Orion-KL. The antenna temperatures are in terms of T_R^* and have not been corrected for beam dilution; we estimate that our calibration scale is correct to within 20%.

Figure 1 shows the H₃O⁺ candidate line at 307,192 MHz at 2 MHz (a) and 1 MHz (b) resolution, along with two unidentified lines and newly detected transitions of several known interstellar species, all of which are reported with line parameters in Table 1. Figure 1 represents a total of more than 7 h of integration time at USB centre frequencies of 307,165 and 307,161 MHz (to de-correlate filters). Complementary integration for 5 h centred on 307,236 MHz showed similar results, and by observing how the spectral lines of the lower sideband (LSB) shifted with respect to the USB centre frequencies, we could identify the sideband of each spectral feature, and thus determine the line rest frequency to within the filter resolution. In all data sets for Orion-KL, the line at 307,192 MHz was evident at $\geq 3\sigma$. The data sets were then frequency-aligned for the USB and averaged, weighted by the integration time and the inverse of the square of the system temperature, to produce the $\geq 5\sigma$ feature shown in Fig. 2 (12 h of integration) at 1 MHz resolution. A similar alignment and averaging for the LSB showed that no LSB spectral feature could account for the USB line at 307,192 MHz. Thus, by these techniques, the reality of the spectral feature at 307,192 MHz was proved.

Assuming that the Orion-KL spectral feature at 307,192 MHz is H₃O⁺, its observed line parameters and calculated transition dipole moment⁹ of 1.44 D yield total column densities $N_T(\text{H}_3\text{O}^+)$ of 7.7×10^{13} and 8.7×10^{13} cm⁻², in the optically thin thermal cases for excitation temperatures of 50 and 90 K, respectively. We point out that the line width may be underestimated due to

the weak intensity of the feature, the possible confusion with the nearby CH₃OH line in the USB and the proximity of the SO line in the LSB. Assuming $N_T(\text{H}_2) = 3 \times 10^{23}$ cm⁻² for Orion¹⁰, a crude estimate of the volume density ratio $n(\text{H}_3\text{O}^+)/n(\text{H}_2) = 2.6 \times 10^{-10}$ and 2.9×10^{-10} can be made for the thermal cases at excitation temperatures of 50 and 90 K, respectively. We estimate a minimum H₂ density of $\sim 10^6$ cm⁻³ for thermalization of the transition to these excitation temperatures. If a density of 10^5 cm⁻³ characterizes the bulk of the H₃O⁺ emitting region, a far lower excitation temperature (~5 K) prevails for the two levels connected by the P(2,1) transition, although the population in the lower level of this transition is probably thermalized at the temperature of the gas. Under

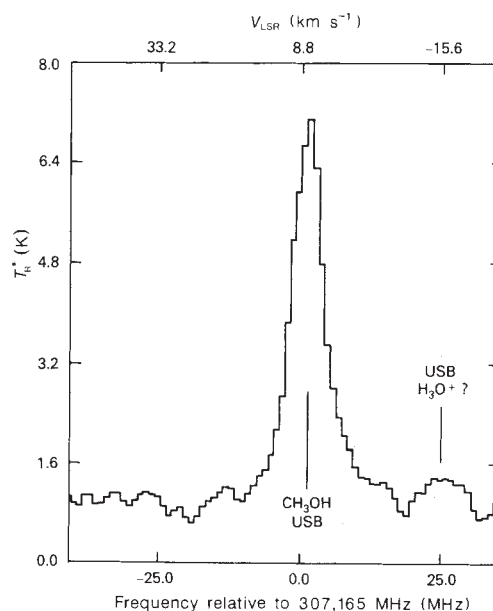


Fig. 2 Hanning smooth spectrum obtained from 12 h of integration towards the Orion-KL nebula with 1 MHz resolution. The lower abscissa is USB frequency relative to 307,165 MHz; the upper abscissa displays the USB velocity scale, assuming a V_{LSR} source velocity of +8.8 km s⁻¹ at a rest frequency of 307,165. The spectrum represents alignment and averaging (see text) of data taken at centre frequencies of 307,161, 307,165 and 307,236 MHz. The CH₃OH line in the USB is blended slightly with a weak H₂CS feature in the LSB for data taken at 307,236 MHz. The $\sim 5\sigma$ feature labelled H₃O⁺? was apparent in each individual data set at $\geq 3\sigma$.

these conditions, for the non-thermal case we obtain $n(\text{H}_3\text{O}^+)/n(\text{H}_2) = 2.3 \times 10^{-9}$ and 2.8×10^{-9} for gas temperatures of 50 and 90 K, respectively. Our thermal and non-thermal sets of fractional abundances are in reasonable agreement with ion-molecule models, which yield 10^{-11} – 10^{-9} , depending on cloud age, gas density and elemental depletion². We have assumed that the volume density ratio can be estimated by the column density ratio. This is not strictly true where fractional abundances change and line saturation effects occur along the line of sight. However, our order-of-magnitude estimate indicates that the spectral feature at 307,192 MHz is roughly consistent with H_3O^+ abundances in present chemical models for Orion-like interstellar clouds.

The estimated fractional abundance of H_3O^+ can be used to determine the fractional abundance of gas-phase H_2O by considering a limited set of ion-molecule reactions⁴. Assuming a rate coefficient of $6 \times 10^{-6} \text{ cm}^3 \text{ s}^{-1}$ for the dissociative recombination reaction of H_3O^+ , a branching ratio of 0.5 to form $\text{H}_2\text{O} + \text{H}$ (ref. 11), rate coefficients of $\sim 10^{-8} \text{ cm}^3 \text{ s}^{-1}$ for the destruction of H_2O by ion-molecule reactions¹², and a value of 0.1 for the ratio of the total abundance of reactive ions to the electron abundance, we obtain fractional abundances for H_2O in Orion of 10^{-5} and 10^{-6} for the non-thermal and thermal cases, respectively.

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Lichtenhahn and Mr Calvin Sparks for assisting with the observations; and Dr Philip Jewell for advice concerning execution of the observations and publication of results. F. C. De L. and E. H. acknowledge the support of NASA grant NAGW-189. NRAO is operated by Associated Universities, Inc., under contract with NSF.

Note added in proof: Independent confirmation of our Orion-KL results was provided by H. A. Wootten (NRAO) and co-workers during 17–21 March 1986. Our laboratory measurements reveal a weak, unassigned (presumably high-excitation) line of CH_3OH at 307,193.27 (5) MHz, which we doubt could account for our Orion-KL line labelled ' H_3O^+ ?', due to line-width, velocity and intensity arguments.

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Impact-induced atmospheres and oceans on Earth and Venus

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High-velocity impacts of planetesimals onto a growing planet result in the impact-degassing of volatiles and the formation of an impact-induced atmosphere. Because of the blanketing effect of such an atmosphere, it is likely that the surface of the proto-Venus melted once the radius exceeded $\sim 40\%$ of the final radius. The final mass of H_2O in the impact-generated atmosphere, predicted to be $\sim 10^{21} \text{ kg}$ on the basis of thermal evolution models of the growing proto-Venus, does not depend on the initial water content of the Venus-forming planetesimals and is almost identical to the present mass of the Earth's oceans. We show here that an impact-induced H_2O atmosphere of $\sim 10^{21} \text{ kg}$ mass probably formed on both Venus and Earth during accretion, but that whereas H_2O in the proto-atmosphere of the Earth could condense to form a hot ($\sim 600 \text{ K}$) ocean, such condensation probably did not occur on Venus.

Recent studies^{1,2} have suggested that an impact-induced atmosphere increases the surface temperature of the Earth to a stage where a magma ocean is possible, and that the total mass of H_2O in an impact-induced atmosphere clusters around 10^{21} kg , a value which is rather insensitive to variations in the input data. As the accretion of planetesimals is fundamental to the process of planetary formation, development of an impact-generated atmosphere during accretion may have occurred on other terrestrial planets. In particular, the early evolution of Venus might be expected to be similar to that of the Earth, because the two planets are of similar size and mass and were formed at comparable distances from the Sun. Why then have Venus and Earth experienced different histories?

Assuming that Venus-forming planetesimals contained H_2O in amounts similar to those retained in Earth-forming planetesimals, we can calculate the evolution of an impact-induced H_2O atmosphere during accretion (see ref. 2 for details of the calculation). Figure 1 shows the atmospheric evolution during accretion for two Venus models. The accretion times, τ_{acc} , are assumed to be $1.6 \times 10^7 \text{ yr}$, which is about one-third of τ_{acc} of the standard Earth model. The degassing parameter and critical pressure for impact dehydration are 0.2 and $2.28 \times 10^{10} \text{ Pa}$, respectively. The solid and dash-dot curves represent the results of the standard model (initial water content of planetesimals $X_w = 0.1\%$) and higher water content model ($X_w = 1\%$), respectively. As shown in Fig. 1, the final amount of H_2O in the atmosphere does not differ greatly between these two models and is $\sim 10^{21} \text{ kg}$. This amount does not vary significantly with changes in X_w and τ_{acc} for $0.03 < X_w(\%) < 1$ and $10^6 < \tau_{\text{acc}}(\text{yr}) < 10^8$ (ref. 2). This is because the solubility of water in silicate melt controls the amount of H_2O in the proto-atmosphere. Note that the final amount of H_2O in the atmosphere is almost identical to the amount presently found in the Earth's oceans ($1.4 \times 10^{21} \text{ kg}$). These are the same features which we found in our model for the Earth². Therefore, we can reasonably consider that both planets originally had proto-atmospheres of similar mass, composed primarily of H_2O . An initial mass of atmospheric water equal to the mass of the terrestrial oceans is also consistent with the present deuterium-to-hydrogen ratio on Venus³.

Given the apparent similarity of the initial atmospheric conditions on Venus and the Earth, it is interesting to study the divergence which subsequently took place in the development of the atmospheres of these two planets. The qualitative nature of the evolution of an impact-generated atmosphere can be inferred simply from its radiative equilibrium thermal structure. The main heat source of such a proto-atmosphere is the impact energy released during accretion. In this case, the atmosphere is heated from below, and thus its thermal structure is determined by the opacity of the atmosphere to infrared radiation (that is, by the blanketing effect). The solar flux supplants the impact energy flux as the main heat source of the atmosphere when its formation is complete, as the Safronov-type accretion rate assumed in this study² decreases rapidly as the final radius is

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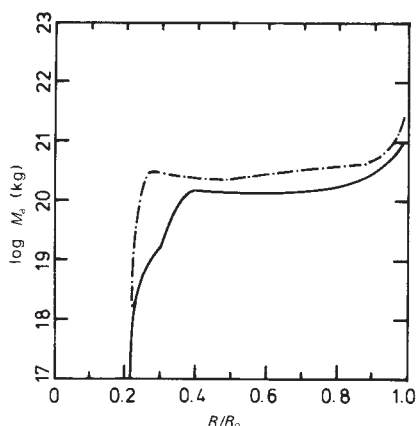


Fig. 1 Total mass M_a of the impact-generated H_2O atmosphere on Venus, plotted against normalized radius for the standard (solid curve) and higher water content (dash-dot curve) models. Note that the final mass of H_2O in the atmosphere is similar to the present mass of the Earth's oceans (10^{21} kg).

approached. When the atmosphere is heated primarily by solar radiation, its thermal structure is determined by the so-called greenhouse effect, which depends on the opacity of the atmosphere not only to long-wave (infrared) radiation, but also to short-wave (visible) radiation. This is because only the solar radiation that is able to penetrate the atmosphere can serve as a heat source. The difference between the blanketing effect and the greenhouse effect is small for a thin atmosphere, because most of the received solar radiation penetrates the atmosphere and heats it from below. For the case of a thick atmosphere, however, most of the solar radiation does not penetrate the atmosphere, as it is scattered and/or absorbed.

We can obtain the temperature profile of a plane parallel atmosphere in radiative equilibrium by solving the equations: $dF_u/d\tau = F_u - \pi B$, $df_u/dt = f_u - \omega(f_u + f_d)/2$, $dF_d/d\tau = -F_d + \pi B$, $df_d/dt = -f_d + \omega(f_u + f_d)/2$ and $F_u - F_d + f_u - f_d = F_0$, with the boundary conditions: $F_u = (1 - A)S_0/4 + F_0$, $f_u = AS_0/4$, $f_d = S_0/4$ and $F_d = 0$ at the top of the atmosphere ($\tau = 0$ and $t = 0$), and $F_u = \sigma T_s^4$ and $f_u = \mu f_d$ at the base of the atmosphere ($\tau = \tau_s$ and $t = t_s$). Here, F and f are the long- and short-wave radiation fluxes, τ and t are the normal optical depths for the long- and short-wave radiation fluxes, B is the Planck function, ω is the albedo for single scattering, σ is the Stefan-Boltzmann constant, F_0 is the thermal energy flux given at the base of the atmosphere, S_0 is the solar flux, A is the planetary albedo, T_s is the temperature at the base of the atmosphere, and μ is the reflectivity of the short-wave radiation at the bottom of the atmosphere. The subscripts u and d denote upward and downward radiation, respectively. For a grey atmosphere, $\pi B = \sigma T^4$, where T is the temperature. To derive the above equations, we assumed local thermodynamic equilibrium for long-wave radiation and isotropic scattering for short-wave radiation, and we used a two-stream approximation. Assuming that $\lambda = \tau/t$ and ω is constant, the solutions are given by

$$\alpha T^4 = F_0 \frac{\tau + 1}{2} + \frac{S_0}{4} \frac{(\lambda + \alpha) + (\lambda - \alpha)\beta + (\alpha^2/\lambda - \lambda)(e^{-\alpha t} + \beta e^{\alpha t})}{(1 + \alpha) + (1 - \alpha)\beta}$$

$$\sigma T_s^4 = F_0 \frac{\tau_s + 2}{2} + \frac{S_0}{4} \frac{[(\lambda + \alpha) - (\lambda - \alpha)e^{-\alpha t_s}](1 - \beta_0 e^{\alpha t_s})}{(1 + \alpha) + (1 - \alpha)\beta}$$

where $\alpha = \sqrt{(1 - \omega)}$, $\beta = \beta_0 e^{-2\alpha t_s}$ and $\beta_0 = [\mu(1 + \alpha) - (1 - \alpha)]/[(1 + \alpha) - \mu(1 - \alpha)]$.

Figure 2 shows the radiative equilibrium thermal structures of the proto-atmospheres of Venus and the Earth, as determined from the above equations. As mentioned previously, the radiative

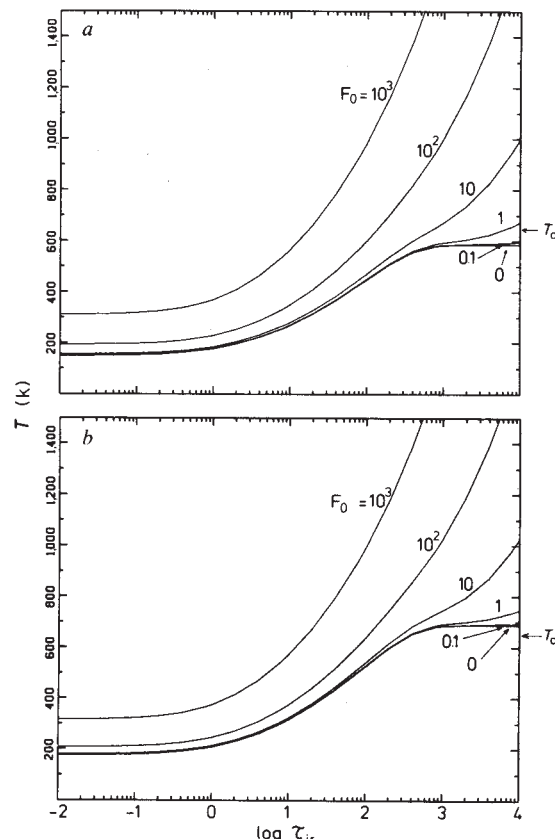


Fig. 2 The radiative equilibrium thermal structures of the proto- H_2O atmospheres for: *a*, the Earth, $S_0 = 960 \text{ W m}^{-2}$; *b*, Venus, $S_0 = 1,830 \text{ W m}^{-2}$. S_0 is the solar flux (we used a value 30% less than the present value), and F_0 is the energy flux released at the surface of a planet (in W m^{-2}). τ_{ir} is the optical depth of the atmosphere to infrared radiation. Note that with a decrease in F_0 the temperature at the base of the Earth's atmosphere becomes lower than the critical temperature T_c for H_2O to liquefy at a pressure of ~ 100 bar.

equilibrium structure depends mainly on the ratio λ of the optical depths of infrared (long-wave) and visible (short-wave) radiation. Although it is difficult to estimate this ratio theoretically and/or experimentally, we may infer it from Pioneer-Venus data on the absorptional features of the venusian atmosphere⁴, where surface pressure is $\sim \frac{1}{4}$ or $\frac{1}{2}$ that of the proto- H_2O atmosphere. The planetary albedo and the net solar radiation flux f_s at the surface are given by $A = [(1 - \alpha) + \beta_0(1 + \alpha)\epsilon^2]/[(1 + \alpha) + \beta_0(1 - \alpha)\epsilon^2]$ and $f_s = \alpha S_0(1 - \beta_0)\epsilon/2[(1 + \alpha) + (1 - \alpha)\beta_0\epsilon^2]$, where $\epsilon = \exp(-\alpha t_s)$. Using the observed values of A (0.77), f_s/S_0 (0.005), S_0 ($2,620 \text{ W m}^{-2}$), T_s (750 K) and μ (0.1) for the present venusian atmosphere⁴, we can determine $\omega = 0.98$ and $\lambda = 31$ from the above equations (ω and λ are almost independent of μ). As the pressure effect on the absorption features of the atmosphere may not change significantly with wavelength, the ratio λ is expected to be insensitive to variations of pressure. The temperature effect on the absorptional features is also small, for temperature variations on the order of several hundred degrees. Therefore, we may consider $10 < \lambda < 100$ for the H_2O proto-atmosphere. In this respect, the following results are insensitive to this ratio. For both planets, the equilibrium thermal structure shifts to a lower level as the impact energy rate decreases. As the impact energy rate approaches zero (greenhouse case), the equilibrium thermal structure for the Earth falls below the vapour pressure, which means that the H_2O in the Earth's proto-atmosphere can condense to a liquid. The equilibrium thermal structure for Venus, however, remains higher than the vapour pressure. It is therefore possible that the H_2O

in the Earth's proto-atmosphere condensed to form oceans, while in the proto-venusian atmosphere it remained in gaseous form. Studies of the escape of H₂O from the proto-venusian atmosphere (see ref. 5) suggest that the H₂O in the proto-venusian atmosphere may have disintegrated into hydrogen and oxygen through photodissociation, with the resulting H₂ subsequently escaping through a hydrodynamic process⁵.

Figure 2 shows that the temperature of the first rain on Earth was ~600 K, and thus a hot proto-ocean may have resulted. The mean temperature of this ocean has been estimated from measurements of the oxygen isotope composition of metamorphosed sedimentary rock. According to Oskvarek and Perry⁶, the mean temperature of the early Archaean ocean may have been as high as 420 K. Our result is consistent with this observation. Sagan and Mullen⁷ noted that the surface temperature of the proto-Earth would have been too cold for water to be liquid if the atmosphere had the same composition as at present; they argued for the existence of a reduced atmosphere, which would lead to warmer conditions on the proto-Earth. Our model, in contrast, does not require such an *ad hoc* assumption. We speculate that the interactive evolution of the hot proto-atmosphere, proto-ocean and proto-crust may have played an important role in the origin of the continents on the Earth; this topic deserves further study.

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The quasi-crystalline phase in the Mg-Al-Zn system

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Shechtman *et al.*¹ have reported a phase in rapidly solidified Al₈₆Mn₁₄ alloy with long-range orientational order, but with icosahedral point-group symmetry, which is inconsistent with lattice translations. However, Pauling² has argued that the apparent icosahedral symmetry in Al₈₆Mn₁₄ alloy is due to directed multiple twinning of cubic crystals with a cube edge of 26.7 Å. We report here the powder X-ray diffraction study of a rapidly solidified Mg₃₂(Al, Zn)₄₉ alloy which shows 5-3-2 symmetry diffraction in transmission electron microscopy (TEM). Also reported is a study of rapidly solidified Mg₂Al₃ alloy. The advantage of these alloys is that β-Mg₂Al₃, which has nearly the same structure as proposed by Pauling for the rapidly quenched Al₈₆Mn₁₄, is an equilibrium phase. This is unlike the Al-Mn system, where, according to Pauling, the cubic phase occurs in a metastable and already twinned form. The present choice of alloy systems makes it possible to compare the X-ray diffraction patterns from the quasi-crystalline phase and a cubic phase with nearly the same structure as proposed by Pauling. We observe that the X-ray diffraction pattern of rapidly

Table 1 Analysis of the X-ray diffraction pattern (Fig. 1c) obtained from rapidly quenched Mg₂Al₃

(hkl)	<i>d</i> _{cal} (Å)	<i>d</i> _{obs} (Å)	<i>I</i> / <i>I</i> ₀	(hkl)	<i>d</i> _{cal} (Å)	<i>d</i> _{obs} (Å)	<i>I</i> / <i>I</i> ₀
(001)	9.65			(031)	1.63		
(010)	4.95			(123)	1.62		
(002)	4.83			(006)	1.61		
(011)	4.40	4.42	2	(115)	1.60		
(012)	3.45			(032)	1.56		
(003)	3.22			(016)	1.53		
(110)	2.86			(124)	1.48	1.48	3
(111)	2.74	2.73	3	(033)	1.47		
(013)	2.70	2.66	3	(220)	1.43	1.43	8
(020)	2.47	2.47	100	(221)	1.41	1.42	4
(112)	2.46			(116)	1.40		
(004)	2.41			(007)	1.38		
(021)	2.40	2.40	78	(130)	1.37		
(022)	2.20	2.20	9	(222)	1.369		
(014)	2.16			(034)	1.361		
(113)	2.13	2.13	3	(131)	1.358		
(023)	1.96	2.00	1	(026)	1.348		
(005)	1.93			(125)	1.343		
(120)	1.87			(017)	1.328		
(114)	1.84			(132)	1.320		
(121)	1.83			(223)	1.306		
(015)	1.80			(133)	1.260		
(122)	1.74			(035)	1.254		
(024)	1.73			(117)	1.240		
(030)	1.65			(040)	1.237	1.239	2

Table 2 Analysis of the X-ray diffraction pattern (Fig. 1a) from rapidly quenched Mg₃₂(Al, Zn)₄₉ alloy

<i>d</i> (Å)	<i>I</i> / <i>I</i> ₀	Icosahedral indices	Observed <i>d</i> (100000)/ <i>d</i>	Theoretical <i>d</i> (100000)/ <i>d</i>
4.23	5	(110001)	0.57	0.56
3.74	4	(111010)	0.65	0.65
2.423	51	(100000)	1.00	1.00
2.292	100	(110000)	1.057	1.051
2.129	6	(561033)	1.138	1.136
2.032	23	(111101)	1.192	1.193
1.778	1	(220001)	1.363	1.358
1.428	19	(101000)	1.697	1.701
1.351	2	(210000)	1.793	1.792
1.224	4	(110010)	1.980	1.973
1.217	1	(200000)	1.991	2.000

solidified Mg₃₂(Al, Zn)₄₉ alloy is distinct from those of the equilibrium phases and that the pattern can be completely indexed to an icosahedral phase. We argue that the above observation is inconsistent with the proposal that the material consists of an aggregate of twinned cubic crystals. Therefore, the alternative is to invoke a quasi-crystalline lattice to explain the observed 5-3-2 symmetry diffraction in TEM.

Although selected area electron diffraction patterns (SADP) displaying 5-fold, 3-fold and 2-fold axes can be explained by multiple twinning of cubic crystals³, Shechtman *et al.*¹ argued that their phase shows long-range orientational order with icosahedral point-group symmetry because the dark-field images taken from any reflection had the entire grain illuminated. Also, micro-diffraction from many different volume elements of a grain (each with an area of ~200 Å²) displayed all reflections in the SADPs. However, their contention in support of the absence of microtwinning, that the X-ray diffraction pattern of rapidly quenched Al₈₆Mn₁₄ alloy cannot be indexed to any Bravais lattice, has been re-examined by Pauling², after including the unindexed lines in a pattern obtained from Schechtman.

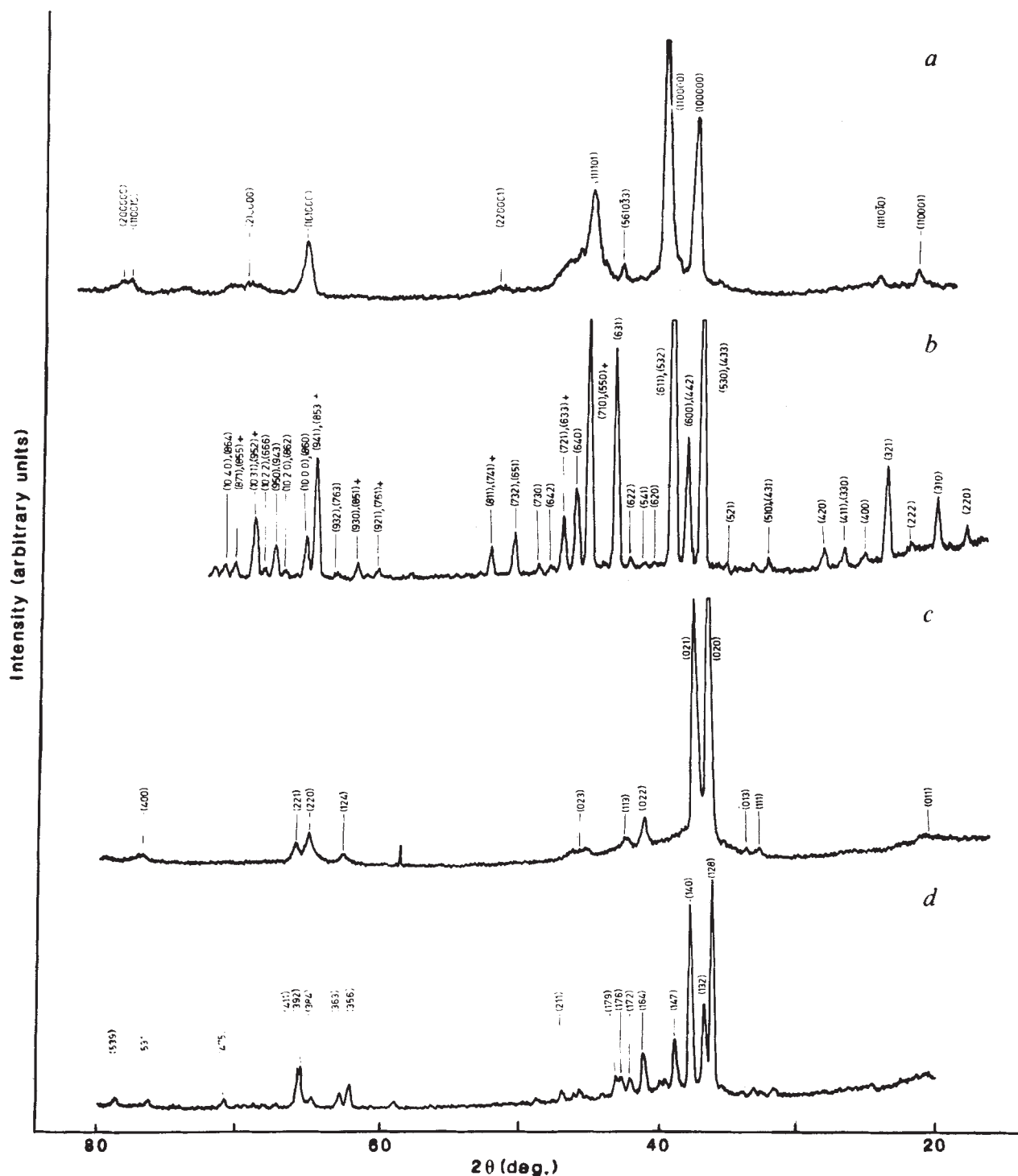


Fig. 1 X-ray diffraction pattern (CuK α radiation) from: *a*, rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ indexed to an icosahedral phase (see text); *b*, rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ after transformation ($cI162$, $a = 14.16 \text{ \AA}$); *c*, rapidly quenched Mg_2Al_3 (hexagonal with $a = 5.73 \text{ \AA}$, $c = 9.54 \text{ \AA}$); and *d*, rapidly quenched Mg_2Al_3 after transformation ($cF1192$, $a = 28.28 \text{ \AA}$).

Pauling re-indexed the entire pattern to a cubic cell with an edge of $26.7 \text{ \AA}$. However, because the proposed cubic cell is large and intensity calculations have not been made, the accuracy of the indexing proposed by Pauling is highly dependent on the reliability of his model for the contents of the unit cell.

Pauling proposed a model in which a molten alloy of $\text{Al}_{86}\text{Mn}_{14}$, on sudden cooling, could form metastable cubic crystals of cube edge $\sim 26.7 \text{ \AA}$, with the unit cube containing $\sim 1,120$ atoms; and that these crystals would show ordered multiple growth such that 20 of them grow out from a central seed to

produce an aggregate with icosahedral symmetry. The arrangement of the atoms in the unit cell is similar to that in the unit cell of rare-gas hydrates. $\beta\text{-Mg}_2\text{Al}_3$, which is cubic with cube edge $28.24 \text{ \AA}$ and 1,192 atoms per cell, has essentially the same structure² as the metastable cubic phase.

In this context, the X-ray diffraction pattern from rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy, which gives 5-3-2 symmetry diffraction patterns in TEM⁴, is of interest. We prepared the alloys $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ (with Al and Zn in the ratio 1:2) and Mg_2Al_3 from the pure elements (better than 99.9% in all cases) by r.f. induction melting over a water-cooled copper hearth. The

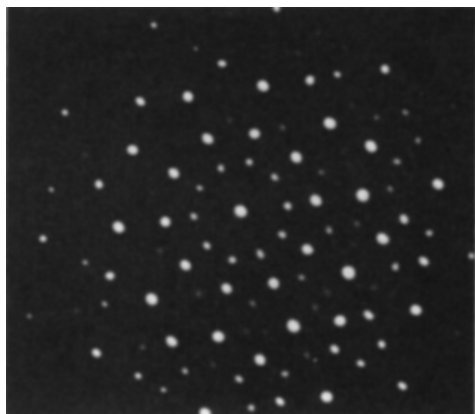


Fig. 2 Selected area diffraction pattern from a typical grain of rapidly solidified $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy displaying 5-fold symmetry.

alloys were re-melted in quartz nozzles and rapidly solidified into thin ribbons by ejecting onto a rotating copper wheel under argon cover. The quenched ribbons were thinned by electropolishing and were examined by TEM. X-ray diffraction patterns were obtained using a Siemens diffractometer with $\text{CuK}\alpha$ radiation.

The X-ray diffraction patterns from rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ and Mg_2Al_3 are shown in Figs 1a and c, respectively. Although there is an apparent similarity in these patterns, the latter could be indexed completely to a hexagonal unit cell with $a = 5.73 \text{ \AA}$, $c = 9.54 \text{ \AA}$ (see Table 1), and its SADPs revealed no axis with 5-fold symmetry. In the case of rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy, the TEM patterns showed 5-3-2 symmetry. The SADP along a 5-fold axis is shown in Fig. 2. The X-ray pattern in Fig. 1a could be completely indexed, following Bancel *et al.*⁵, using the 12 vectors pointing to the vertices of an icosahedron, which are generated by cyclic permutation of $(q_x, q_y, q_z) = (\pm 1, \pm \tau, 0)$. The six independent vectors chosen are $q_1 = (1, \tau, 0)$, $q_2 = (1, -\tau, 0)$, $q_3 = (0, 1, \tau)$, $q_4 = (0, 1, -\tau)$, $q_5 = (\tau, 0, 1)$, $q_6 = (-\tau, 0, 1)$, where τ is the golden mean, $(1 + \sqrt{5})/2$. The observed d -spacings and the icosahedral indices are given in Table 2.

Differential scanning calorimetric (DSC) scans of as-quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ and Mg_2Al_3 showed well-defined exotherms with peak temperatures of 613 and 568 K, respectively (Fig. 3). The ribbons were annealed at temperatures beyond the exotherms for 15 min in the DSC cell in argon atmosphere. The annealed specimens showed no axis with 5-fold symmetry in TEM.

X-ray diffraction patterns were obtained from the annealed specimens in identical conditions as for the as-quenched alloys. The patterns obtained from annealed $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ and Mg_2Al_3 are shown in Fig. 1b and d, respectively. The former can be indexed to the equilibrium cubic phase ($cI162$, $a = 14.16 \text{ \AA}$; ref. 6) and the latter to $\beta\text{-Mg}_2\text{Al}_3$ ($cF1192$, $a = 28.3 \text{ \AA}$; ref. 7).

A comparison of the diffractograms in Fig. 1a, b, for the rapidly quenched and annealed $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy, respectively, suggests that if we were to assume that the 5-fold symmetry in TEM results from 20 cubic crystals with definite angular relationships in their orientations, the obvious choice for the cubic unit cell will be the $cI162$ type. The reason for this is that

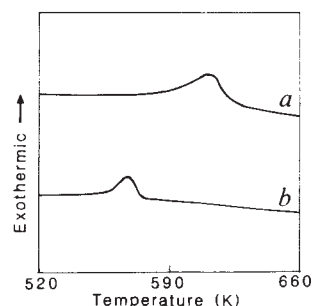


Fig. 3 DSC thermograms from rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ (a) and Mg_2Al_3 (b).

the major lines in Fig. 1b coincide with the lines in Fig. 1a. The rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy shows a distinct transformation temperature in DSC. This transformation could be a crystal structure change or an irreversible change in which the cubic crystals lose their mutual angular relationships. However, only a crystal structure change can be expected to cause a substantial change in the X-ray powder diffraction patterns. The X-ray diffraction pattern in Fig. 1b shows many intense lines which are missing altogether in Fig. 1a. Therefore, the exotherm in the DSC thermogram does not correspond to a transformation in which the mutual angular relationships between the cubic crystals are destroyed. As most of the lines in Fig. 1a have d -spacings in common with those in Fig. 1b, the question of texture in the as-quenched ribbons also arises. However, a comparison of diffractograms obtained from the strips and a fine powder made from the strips ruled out texture in the as-quenched ribbons as a cause for there being relatively fewer lines in Fig. 1a. Note also that the diffraction pattern in Fig. 1a is not similar to that of $\beta\text{-Mg}_2\text{Al}_3$ in Fig. 1d.

Thus, the rapidly quenched $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ alloy does not consist of aggregates of cubic crystals of either type, $cI162$ or $cF1192$. The X-ray pattern is completely indexable to an icosahedral phase. Hence, a quasi-crystalline lattice is necessary to explain the observed 5-3-2 symmetry diffraction in TEM.

Pauling (personal communication) has suggested that the pattern given in Fig. 1a can be indexed to a disordered NaCd_2 -type structure with $a = 27.82 \text{ \AA}$ ($cF1120$), and that on annealing there are changes in the structure such as to eliminate all or most of the randomness. However, it is not clear as to how, on annealing, the material transforms to a $cI162$ -type structure rather than to an ordered form of NaCd_2 -type structure. The indexing of the pattern in Fig. 1a to a disordered NaCd_2 -type structure is not necessarily unique. One could also assign the pattern to a $cI162$ -type phase with disorder in the unit cell, as the major lines in Fig. 1b coincide with the lines in Fig. 1a. However, there has been no report of a disordered $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ -type structure. The equilibrium $\text{Mg}_{32}(\text{Al}, \text{Zn})_{49}$ phase has a unit cell in which spherical aggregates of atoms which basically have 5-3-2 symmetry are packed in body-centred cubic lattice positions. The question of whether disorder in such a lattice would lead to a quasi-crystalline structure, yielding the X-ray diffraction pattern shown in Fig. 1a, becomes interesting.

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High-resolution transmission electron microscopy of silicon re-growth at controlled elevated temperatures

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Direct observations of atomic surface rearrangements and dislocation reactions using high-resolution transmission electron microscopy (TEM) techniques have been described for gold and for cadmium telluride¹⁻⁶. A major disadvantage of these experiments has been that they rely on the heating effect of the imaging electron beam to raise the temperature of the sample so that thermally activated processes can be induced. This severely restricts the materials which can be studied and significantly reduces the control of the observer in bringing about changes of interest. We have previously suggested⁴ that many solids would be open to such investigation if an appropriate temperature range could be reached. Using a simple Arrhenius kinetic law, we estimated, for example, that silicon would need to be held at $\sim 600^\circ\text{C}$ to make equivalent observations to those seen in CdTe. Recognizing the contemporary technological applications of silicon, such a step would be important in fundamental studies of device circuits. We report here that this type of imaging can indeed be performed successfully, without recourse to specialized instrumentation, and that new insights can be gained concerning defect production and phase transformations in this important material.

The experiment involved the observation of silicon re-growth *in situ* in a TEM at temperatures between 500 and 800 °C. A silicon thin film (300 nm) had been epitaxially deposited on a sapphire substrate and had undergone a dual ion-implant and annealing treatment. The implants have the effect of rendering the highly defective silicon crystal amorphous, while the annealing produces a virtually defect-free single-crystal film⁷. We investigated this latter reaction (the amorphous to crystalline transformation) during solid-phase epitaxial re-growth.

Samples were made for TEM using a standard cross-sectioning technique⁸, which allowed high-resolution electron microscopy (HREM) imaging in the standard [110] orientation. The electron microscope was a conventional Philips EM400 ST operating at 120 kV, with a resolution of ~ 0.32 nm. Heating was achieved with the standard, commercially available holder (model number PW 6592), in which an electrical feed-through heats a platinum pad on which was placed the 3-mm disk specimen. The temperature is measured by a thermocouple attached to the pad. Because of the high thermal conductivities of sapphire and silicon, the thermocouple reading should be

reasonably close to the actual specimen temperature. Care was taken to use reproducible imaging conditions so that any beam-heating or other effects would give rise to a systematic error in the apparent temperature.

One of the most important experimental parameters for the HREM is the specimen orientation with respect to the imaging electron beam. As the present holder has only single-tilting capability, the external configuration must be used to facilitate orientation control. The surface normal of the silicon film is [100]. In a cross-section specimen, the thin film is easily seen as a line across the middle of the disk. When this line is oriented perpendicular to the holder rod, the tilt axis is then accurately the [100] crystal direction. Appropriate sectioning therefore allows attainment of the desired orientation. Axial crystal lattice images were produced using the transmitted and six diffracted beams. A Philips plumbicon image pick-up system (model number PW 6326/50), coupled to a standard video recorder and television monitor, was used to display and record the pictures. Subsequent image processing was carried out with a Quantex QX-9000 digital image processing and analysis system; additional details are given elsewhere⁹. Starting from room temperature, it takes ~ 1 h for the image drift to stabilize sufficiently for reasonable HREM observations to be obtained at $\sim 700^\circ\text{C}$. The thermocouple reading can be maintained to within $\pm 5^\circ\text{C}$ over several hours, and this is confirmed by the stability of the image itself. Controlled temperature changes can be effected, although a few minutes are required for re-stabilization.

Figure 1 shows a region of the crystalline/amorphous silicon interface. A photograph taken from continuous play-back of the video recording (Fig. 1a) is compared with the digitally enhanced image (Fig. 1b). The former image is of lower quality than conventional HREM images photographically recorded at room temperature; however, the processed image is adequate for standard image interpretation. Detailed studies of the kinetics of the crystallization process as a function of temperature yielded a variation and an activation energy consistent with those found by two alternative techniques, indicating that the same mechanism is operative¹⁰. Our data show a small systematic displacement from the others which may arise either from an error of $\sim 40^\circ\text{C}$ in the apparent temperature or from an influence of the TEM thin foil on the reaction rate.

Crystallization is observed to occur by the gradual advance of the crystalline/amorphous interface; there is no detectable crystal nucleation ahead of this interface. Faceting on low-index planes is common, particularly on {100} (which is the broad face of the epitaxial growth) and {111}. The latter give rise to protrusions, or recessions, of the growth front, as shown in Fig. 1. One peculiarity, which cannot be detected by bulk investigative techniques, is that occasionally the interface would advance over several atomic distances apparently instantaneously (that is, between two successive 1/30 s video frames). Thus, a large

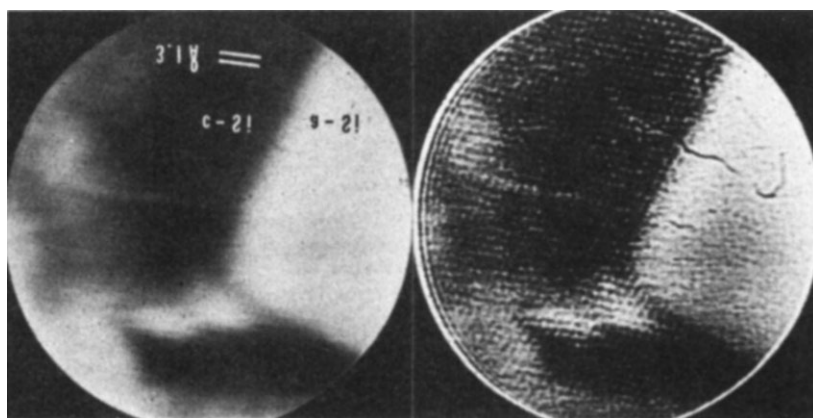


Fig. 1 Comparison of video-recorded lattice images of the amorphous/crystalline silicon interface at 720 °C: a, 1-s exposure photograph during play-back of the video-tape; b, photograph of the image after digital filtering and eight-frame averaging.

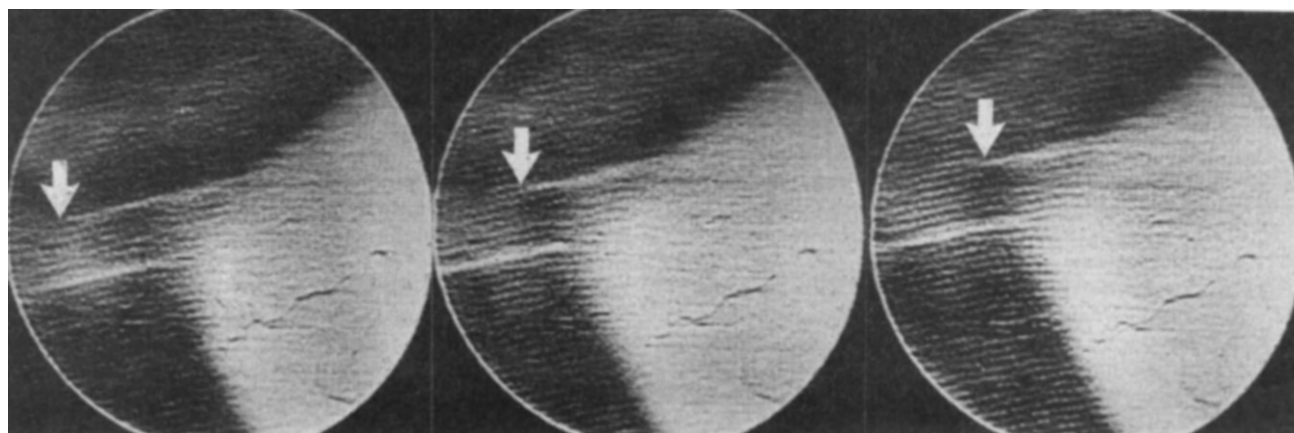


Fig. 2 Sequence of micrographs at 720 °C, taken 1 s apart, showing sudden crystallization of many atoms, advancing the crystalline/amorphous interface. Frame-by-frame analysis shows that the event occurs between two successive frames, 1/30 s apart.



Fig. 3 Sequence of micrographs at 720 °C, taken 10 s apart, showing the gradual motion of a Shockley partial dislocation (arrowed) towards the amorphous/crystalline interface, annihilating a stacking fault in the process. This sequence is similar to that observed previously in CdTe (refs 5, 6).

number of atoms, in some cases $>10^4$, extend the epitaxial crystal spontaneously. This suggests that under certain conditions the mechanism of crystallization occurs by something other than a simple atom-by-atom process, and that the silicon tetrahedral network can be created perfectly with much coordination of the atomic rearrangement. Such cooperative phenomena would be expected in a heterogeneous nucleation event. This behaviour is illustrated by the sequence shown in Fig. 2, from growth at a measured temperature of 720 °C.

Also unexpected was the observation that the interface sometimes regresses. This can be explained if we invoke the influence of interfacial stresses (arising from the volume change of the transformation), as well as the influence of interfacial surface energies for the differing crystallographic orientations, as playing some part in the behaviour. We speculate that these video-recordings of the regressions and advances of the interface are the first direct record, at near-atomic resolution, of the fluctuations expected from transition-state theory between a metastable and a stable phase. The presence of oxygen and aluminium (from the sapphire), as well as the thinness of this particular region, may also have some influence on the fluctuations.

Lattice imperfections can also be created at the interphase interface, which lends credence to the idea that significant stresses are present, particularly at 'corners' like that shown here. Shockley partial dislocations can be ejected, passing along a {111}-type slip plane and resulting in an intrinsic stacking fault. More complex dislocation reactions can occur which can be analysed in detail by Burgers circuits drawn around the

dislocation cores at various stages of the reaction. Although we observed several different events, the image quality was not sufficiently good for the analysis to be carried out with real certainty in all cases. However, this should not be a problem with the new generation of high-resolution TEMs now available. Figure 3 shows a sequence in which a Shockley partial is approaching the interface and recreating the perfect crystal, an observation identical to that made in cadmium telluride, described previously^{5,6}. One has the impression that the interface region is unstable, and that unusual events can occur at any instant. Furthermore, although stacking faults in many materials are thought to arise from accidents of growth, we see here that they are directly created by a dislocation mechanism.

We have been successful in obtaining lattice-resolution micrographs and continuous recordings at controlled elevated temperatures, using a conventional TEM and heating stage. We have studied crystallization and defect reactions in silicon at a temperature (~ 700 °C) similar to that referred to in our original predictions⁴ (that atomic rearrangements would occur in silicon at ~ 600 °C), and the phenomena are exactly analogous to those observed in CdTe using beam heating^{5,6}. Therefore, it is reasonable to conclude that atomic-level events of this type, including phase transformation processes, may be investigated in detail in many materials, at the appropriate temperature.

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A new type of host compound consisting of α -zirconium phosphate and an aminated cyclodextrin

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Pillaring of layered compounds by polynuclear hydroxy metal cations or bulky organic species has been applied to the formation of microporous networks analogous to zeolites^{1,2}. Similar types of compound may be obtained by the intercalation of host molecules such as cyclodextrins in layered parent hosts. Our previous work^{3,4} first demonstrated such a combined host compound, in which molecules of mono-(6- β -aminoethylamino-6-deoxy)- β -cyclodextrin (CDen) are intercalated as a bi-layer with their cavity axes perpendicular to the silicate layers of Cu(II)-montmorillonite. We have now prepared a complex of layered α -zirconium phosphate with CDen, in which the CDen molecules are arranged as a bi-layer but with their cavity axes parallel to the inorganic layers. The dextrin moiety of the present complex also appears to possess a zeolitic character.

Cyclodextrins contain a cylindrical cavity capable of including a variety of molecules, together with catalytically active hydroxyl groups. The cyclic dextrins and their derivatives therefore serve as micro-encapsulating agents for drugs or as models of enzymes⁵. Zirconium phosphate is an inorganic ion exchanger with a layered structure and acts as an intercalating agent for polar organic substances⁶. The crystalline phase with composition $\text{Zr}(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ has an inter-layer spacing of 7.6 Å and is called α -zirconium phosphate (α -ZrP).

The α -ZrP sample was the same as that used previously⁷; the CDen was prepared as described in refs 3, 4. Crude CDen was purified by chromatography, using a carboxymethyl cellulose column with 0.1 M ammonium bicarbonate as an eluant, followed by repeated evaporation of the eluate in the presence of a small amount of ammonia. The phosphate sample was soaked in an aqueous solution containing varying amounts of CDen at 25 °C for 14 days, centrifuged, fully washed with water, and air-dried at 40 °C.

Figure 1 shows the X-ray diffraction patterns of the resulting solids. As a result of the addition of CDen, the peak at $d = 7.6$ Å attributable to the (002) reflection of α -ZrP decreased considerably in intensity, while a new diffraction peak (C(001)) appeared at $d = 35.6$ Å along with its higher-order counterparts. This indicates that the α -ZrP phase is directly converted into its CDen complex with an inter-layer spacing of 35.6 Å. This con-

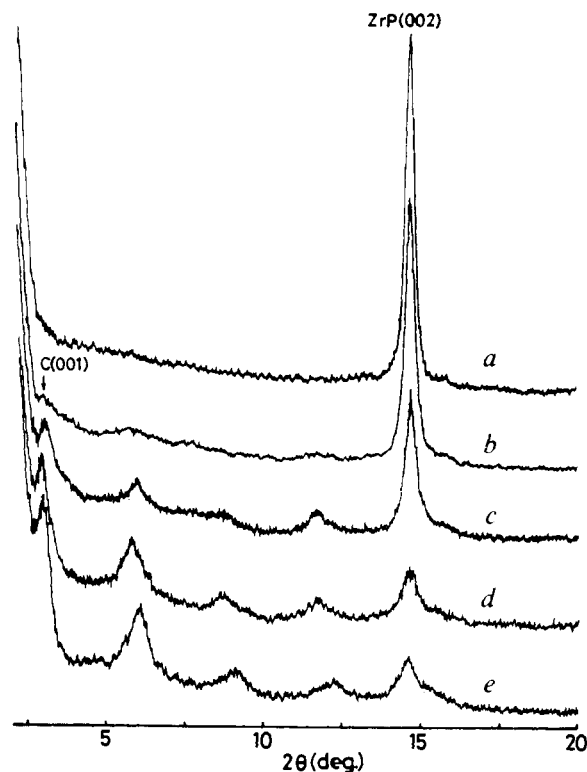


Fig. 1 X-ray diffraction patterns of α -zirconium phosphate (a) and its complexes with CDen (FeK α radiation). CDen addition levels (mmol g^{-1}): 0.43 (b), 1.3 (c), 2.3 (d) and 5.2 (e).

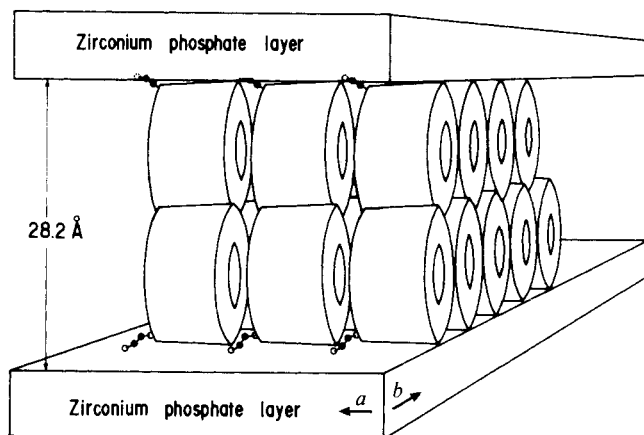


Fig. 2 Model proposed for the packing of CDen molecules in the inter-layer space of α -zirconium phosphate. Directions *a* and *b* refer to the axes of the α -ZrP unit cell (see Fig. 3).

version proceeded with increasing amounts of CDen until, at a level of 5.2 mmol CDen per g α -ZrP, the rate of conversion ξ amounted to 88%, according to a rough estimate based on the peak area of the (002) reflection for the residual phosphate phase. The amounts of CDen and water, *x* and *y*, per formula weight of α -ZrP for the solid obtained at this highest level of CDen addition (sample A) were determined to be 0.368 and 5.3 mol, respectively, by thermo-gravimetric analysis.

Similarly to basic amino acids⁸, CDen is likely to be intercalated by α -ZrP in such a way that the inter-layer surface protons are replaced by the terminal NH_3^+ cations. The thickness of the intercalated layer, Δ , for the resulting CDen-ZrP complex is estimated as 28.2 Å by subtracting 7.4 Å for the thickness of the inorganic layer from the observed inter-layer spacing. It is therefore reasonable to assume that CDen molecules, each 15.4 Å in

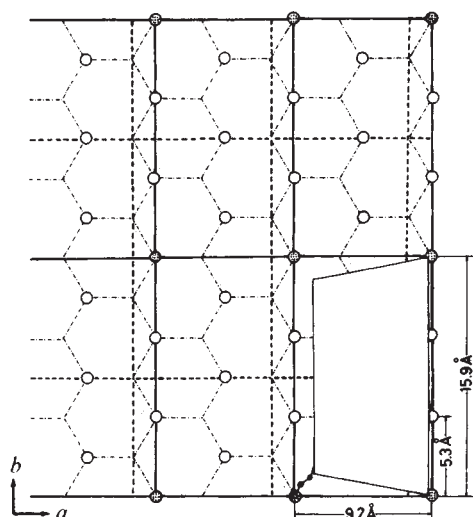


Fig. 3 An idealized relationship between the arrangements of the POH groups and CDen molecules in the inter-layer space of α -zirconium phosphate (projection on the a - b plane). The upward-pointing POH groups in the lower layer are represented by circles and the downward-pointing ones in the upper layer are located at the triple points of dash-dot lines. Each CDen molecule with its cavity axis parallel to the a axis anchors through the basic terminus to the POH site (shaded for the lower layer) and is placed in the rectangle surrounded by the solid (lower layer) or broken (upper layer) lines.

diameter and 8.0 Å in the thickness of the torus, are intercalated as a bi-layer with their cavity axes perpendicular or parallel to the phosphate layers. Assuming hexagonal close packing of CDen molecules in a plane perpendicular to their cavity axes, the effective area per molecule is evaluated as $2\sqrt{3} \times (15.4/2)^2$ or 205 Å². The effective area per POH site in α -ZrP is 24.3 Å² (ref. 7). Hence, the maximum values of x and Δ for the perpendicular arrangement can be calculated, respectively, as $2 \times 24.3/205 = 0.24$ and as twice 11.8 Å, which is the sum of the thickness of the torus and the extended chain length of the aminoethylamino group, or 23.6 Å. These calculations are not consistent with the observed data. On the other hand, the parallel arrangement yields a maximum x value of $2 \times 24.3/(15.8 \times 8.0) = 0.40$ and a minimum Δ value of $15.4(1 + \sqrt{3}/2) = 28.7$ Å, in fairly close agreement with the observations. Thus, we propose the model shown in Fig. 2 as the probable arrangement of CDen molecules in the inter-layer space of α -ZrP. This model gives a ξ value for sample A of $0.368/0.40 = 92.0\%$, which is close to the value roughly estimated from the X-ray observations. It can also be assumed that sample A is a mixture of 92 mol% of a complex, $\text{Zr}(\text{HPO}_4)_2(\text{CDen})_{0.4} \cdot 5.7\text{H}_2\text{O}$, and 8 mol% of α -ZrP.

Referring to the crystal structure of α -ZrP, the POH groups pointing up or down in each layer are located in a monoclinic cell with dimensions of 9.2 and 5.3 Å along the a and b axes, respectively⁶, as shown in Fig. 3. Any two adjacent layers of ZrP are staggered in such a way that the downward-pointing POH groups in the upper layer are shifted by 3.07 Å along the a axis relative to the layer below. It is therefore conceivable that the first intercalated layer of CDen molecules, with their cavity axes parallel to the a axis of the α -ZrP crystal, anchors through the basic termini to every POH site, at 9.2-Å intervals, in every sixth row, 15.9 Å apart, along this axis in the ZrP layer below. The second intercalated layer is probably bonded in the same manner to the ZrP layer above, but is shifted by 7.7 and 8.0 Å along the a and b axes, respectively, relative to the first layer. Thus, the parallel bi-layer model suggested above is also consistent with the geometrical arrangement of POH sites at the inorganic layer surface.

Depending on the size and ionic or molecular character of

the substrate, the cyclodextrins and their adducts are crystallized as empty molecules or as inclusion complexes, with channel or cage structures in which the cyclodextrin molecules are stacked like coins in a roll or arranged in a herring-bone pattern⁵. The channel structure is formed only as inclusion complexes with long-chain molecules such as poly-iodine. As the hydrate of β -cyclodextrin contains 7 water molecules within the cavity⁹, it is probable that $0.4 \times 7/5.3 = 49\%$ of the water molecules in sample A are located in the cavity of the intercalated CDen molecules. According to thermo-gravimetric observations, this sample released 85 mol% of its water during heating to 120 °C, and the dehydrated solid re-adsorbed 92 mol% of the desorbed water by exposure to saturated water vapour at 25 °C, followed by air-drying under the same conditions as for sample A. These preliminary results and the above geometrical considerations suggest that the CDen molecules in one intercalated layer are arranged with their cavity axes parallel to the inter-layer surface, either in alignment to form empty channels, 6 Å in diameter, or, if displaced, with bottlenecks large enough to allow water molecules 2.65 Å in diameter to diffuse. Thus, the dextrin moiety of the CDen-ZrP may possess a zeolitic character, at least for small molecules such as water and hydrogen, although definite conclusions must await further work. Complexes of α -zirconium phosphate with CDen or other cyclodextrin derivatives may prove useful as solid supports in gas or liquid chromatography and as micro-encapsulating agents for various substances such as drugs.

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Plume versus lithospheric sources for melts at Ua Pou, Marquesas Islands

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The remarkable distinction between the compositions of ocean island basalts (OIBs) and mid-ocean ridge basalts (MORBs) provides an important constraint on models of mantle composition and structure¹⁻³. Previous studies of OIBs⁴⁻⁸, however, have emphasized regional isotopic variations, often relying on a small number of samples from many separate volcanoes. Although this type of sampling has now established the basic range of isotopic variations, with the notable exception of the Hawaiian Islands^{6,9,10}, there is little information on either spatial or temporal variations within a single volcano. Here we report isotopic (Sr, Nd and Pb) and K-Ar age measurements for tholeiites, alkali basalts and differentiated rocks from the island of Ua Pou. In this island volcanism spanned the interval from 5.6 to 1.8 Myr, with the ratio of highly to moderately incompatible trace elements increasing

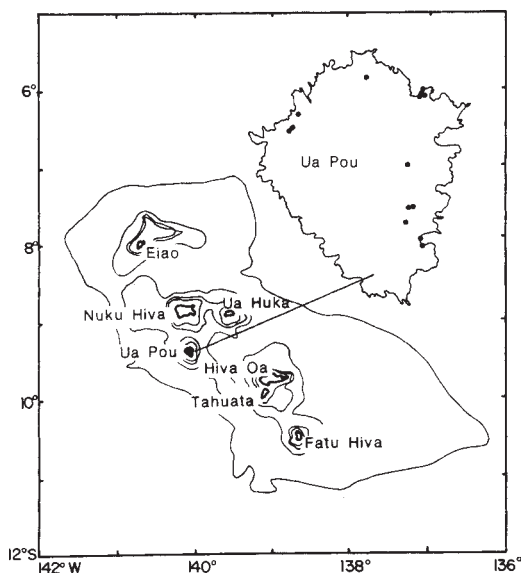


Fig. 1 Map of the Marquesas Islands, an age-progressive volcanic lineament in the south-central Pacific basin, showing the island of Ua Pou. Solid circles (on the enlarged map of the island) mark the location of samples (tholeiites, alkali basalts and differentiates) analysed in the present study.

with time; however, in contrast to Hawaii, $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{207}\text{Pb}/^{204}\text{Pb}$ increased while $^{143}\text{Nd}/^{144}\text{Nd}$ decreased from the tholeiitic to alkalic magmas. The total variation in isotopic composition within this single island is nearly as great as within the entire French Polynesian region^{8,11,12}, and argues against systematic geographical correlations¹³.

The island of Ua Pou, at $9^{\circ}24' \text{ S}$, $140^{\circ}04' \text{ W}$, is one of a chain of more than 20 volcanic islands and seamounts which constitute the Marquesas archipelago in the south-central Pacific Basin (Fig. 1). These volcanoes are linearly arrayed in a WNW-ESE direction, sub-parallel with other young intra-plate Pacific island chains. This characteristic geometry and the reported southeasterly progression in volcano ages^{14,15}, from 6.3 to 1.4 Myr, has linked Marquesan volcanism to a hotspot origin. The abyssal ($>4,000 \text{ m}$) ocean floor on which these islands were constructed formed at the Galapagos Rise (ancestral East Pacific Rise) between 50 and 60 Myr (ref. 16).

Ua Pou hosts a particularly wide range of rock compositions, from tholeiitic to alkalic basalts; the latter have undergone low-pressure fractionation towards trachytic and phonolitic rocks^{17,18}. Although tholeiites are the main rock type in the Hawaiian islands¹⁹, they have not been reported elsewhere in the French Polynesian region; their occurrence in the initial stage of volcanism at Ua Pou suggests that tholeiitic eruptions may occur throughout this region, but may be almost totally confined to submarine portions of the volcanoes.

We report here age determinations for rocks from Ua Pou. Previous age studies¹⁴ on other islands from the Marquesas chain have documented an age progression in volcanism from north-west to south-east at a rate of $\sim 10.4 \text{ cm yr}^{-1}$ (ref. 9). From this regular age distribution along the lineament, the expected age range at Ua Pou is 4.0–2.7 Myr. Re-examination²⁰ of the published age data showed that at the islands of Nuku Hiva and Hiva Oa dated rocks defined two age groups separated by about the same timespan, 0.6 Myr. Furthermore, at each island the older group was predominantly olivine tholeiite while the younger group was alkali basalt. To investigate this apparent petrogenetic evolution at individual Marquesan volcanoes, additional sampling was undertaken by one of us (H.G.B.).

Ua Pou does not exhibit the central collapsed caldera commonly seen at other Marquesas Islands; instead, the island's

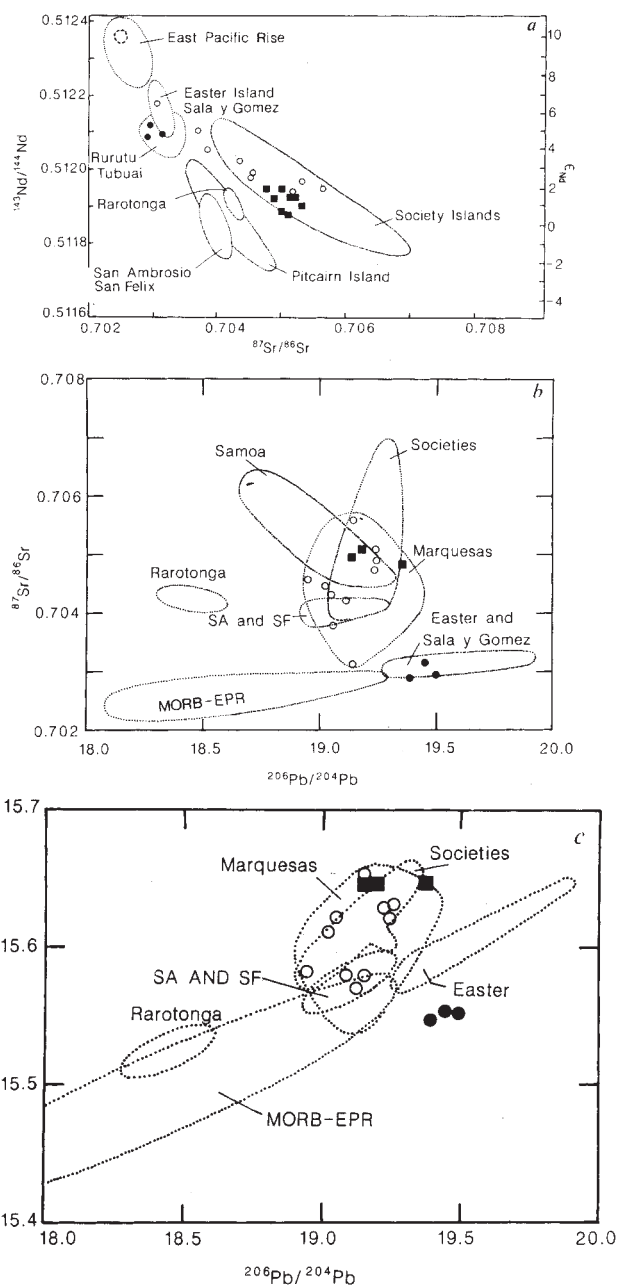


Fig. 2 Isotopic compositions of lavas from Ua Pou compared with those for other basaltic rocks from the south-central Pacific basin. a, $^{143}\text{Nd}/^{144}\text{Nd}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$; b, $^{87}\text{Sr}/^{86}\text{Sr}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$; c, $^{207}\text{Pb}/^{204}\text{Pb}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$. Fields of data are from ref. 25 for islands and East Pacific Rise, except Rurutu and Pitcairn Islands³⁰. Solid circles and squares are tholeiites and alkali basalts, respectively, from Ua Pou. Open circles are other analysed samples from the Marquesas Islands¹². SA, San Ambrosio; SF, San Felix; EPR, East Pacific Rise.

centre is a complex zone of high-level intrusions of trachytic and phonolitic rocks which have obscured the early shield structure of the volcano. Shield lavas outcrop around the perimeter of the island, in stream valleys, in road cuts and along wave-cut cliffs. The samples analysed in this study are well distributed, coming from the northwestern, northern and eastern sides of the island (Fig. 1).

Initial studies¹⁸ of trace element compositions of tholeiites and alkali basalts collected from Ua Pou revealed striking differences in the inferred source compositions for the two groups. Rocks which plot as tholeiites in a standard alkalis versus silica diagram predate the alkali basalt assemblage. The

Table 1 Sr, Nd and Pb isotopic compositions and K-Ar ages for volcanic rocks from Ua Pou, Marquesas Islands

Samples	Rock type	K (%)	Rb (p.p.m.)	Sr (p.p.m.)	Sm (p.p.m.)	Nd (p.p.m.)	$^{87}\text{Sr}/^{86}\text{Sr}$	$^{143}\text{Nd}/^{144}\text{Nd}$	ϵ_{Nd}	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$	K-Ar age (Myr) $\pm 1\sigma$
UAP-011	Tholeiite	0.554	15	548	10.3	43.3	0.70289 $\pm$ 2	0.512070 $\pm$ 12	+4.6	19.50	15.54	39.15	4.46 $\pm$ 0.07
UAP-017	Tholeiite	0.204	3	434	10.0	40.3	0.70294 $\pm$ 4	0.512118 $\pm$ 16	+5.5	19.39	15.54	38.99	4.51 $\pm$ 0.14
UAP-024	Tholeiite	0.651	11	610	13.0	57.6	0.70318 $\pm$ 5	0.512084 $\pm$ 24	+4.8	19.45	15.55	39.01	5.61 $\pm$ 0.06
UAP-001	Alkali basalt	0.871	41	985	11.4	71.4	0.70500 $\pm$ 5	0.511944 $\pm$ 27	+2.1				2.75 $\pm$ 0.03
UAP-002	Alkali basalt	0.554	76	904	11.8	62.4	0.70497 $\pm$ 5						2.78 $\pm$ 0.03
UAP-003	Alkali basalt	0.855	40	972	11.3	61.5	0.70482 $\pm$ 5						2.70 $\pm$ 0.06
UAP-010	Alkali basalt	1.039	145	1,235	13.3	76.0	0.70509 $\pm$ 5	0.511867 $\pm$ 14	+0.6	19.18	15.65	39.31	2.70 $\pm$ 0.04
UAP-026	Alkali basalt	0.524	84	910	11.5	60.7	0.70497 $\pm$ 5	0.511876 $\pm$ 20	+0.8	19.14	15.64	39.20	2.88 $\pm$ 0.08
UAP-012	Tephrite	2.640	116	1,373	15.3	84.6	0.70515 $\pm$ 5	0.511918 $\pm$ 10	+1.6				2.24 $\pm$ 0.05
UAP-015	Hawaiite	2.643	241	1,358	7.9	48.6	0.70481 $\pm$ 5	0.511907 $\pm$ 12	+1.4	19.36	15.65	39.35	1.78 $\pm$ 0.03
UAP-025	Mugearite	4.276	72	1,282	13.0	52.2	0.70531 $\pm$ 6	0.511891 $\pm$ 25	+1.1				2.49 $\pm$ 0.03
UAP-004	Trachyte						0.70508 $\pm$ 5						
UAP-019	Phonolite	5.693	240	146	15.3	108.0	0.70497 $\pm$ 4	0.511917 $\pm$ 22	+1.6				2.42 $\pm$ 0.04
UAP-037	Phonolite	5.887	235	136	6.9	52.8	0.70474 $\pm$ 5						2.42 $\pm$ 0.03

$^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are normalized to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$ and $^{146}\text{Nd}/^{142}\text{Nd} = 0.636151$, K-Ar ages were calculated using the following decay and abundance constants: $\lambda_e = 0.581 \times 10^{-10} \text{ yr}^{-1}$; $\lambda_\beta = 4.963 \times 10^{-10} \text{ yr}^{-1}$; $^{40}\text{K}/\text{K} = 1.167 \times 10^{-4} \text{ mol mol}^{-1}$ respectively. For the standard NBS 987, $^{87}\text{Sr}/^{86}\text{Sr} = 0.71023$ and for BCR-1 $^{143}\text{Nd}/^{144}\text{Nd} = 0.511833$. Rb, Sr, Sm and Nd concentrations are from ref. 18.

tholeiitic phase of volcanism (5.6–4.5 Myr) is separated from the alkali basalt eruptions (2.9–2.7 Myr) and subsequent flows and intrusions of liquids evolved by high-level fractional crystallization (2.5–1.8 Myr). The entire age range of subaerial volcanism at Ua Pou is ~ 3.8 Myr (Table 1). The apparent 1.6-Myr hiatus between the tholeiitic and alkalic stages of volcanism could be the result of incomplete sampling and may be reduced by future geochronological studies on well-mapped sections. The progression from tholeiitic to alkalic volcanism at Ua Pou and its likelihood at the neighbouring islands of Nuku Hiva and Hiva Oa²⁰ is reminiscent of the volcanic evolution of the Hawaiian Islands¹⁹. In the Marquesas Islands, however, no very undersaturated, post-erosional eruptions have followed the alkali basalt stage. It is probable, therefore, that each of the Marquesas islands evolved through a common sequence. Initially, eruptions may have been a mixture of tholeiitic and alkalic compositions, as seen at Macdonald²¹ seamount at the southeastern end of the Austral Islands, and Loihi seamount²² in the Hawaiian Islands. Tholeiitic compositions dominated the early shield-building phase, which at Ua Pou barely reached sea level. This was followed by eruptions of alkali basalt and, later, highly evolved rocks. It is not yet known whether the change from tholeiites to alkali basalts occurred as a gradual transition or as a sharp compositional break after a significant hiatus in volcanic activity.

Major and trace element concentrations for Ua Pou rocks have been reported elsewhere^{17,18}. Liotard *et al.*¹⁸ identified tholeiitic rocks at many of the Marquesas Islands and, in particular, quartz-normative tholeiites at Ua Pou, in addition to the earlier described alkali basalts. The tholeiites and alkali basalts cannot be related to one another by either variable partial melting from a common source or fractional crystallization from a parental melt, because of many clear differences in their trace element patterns. Both groups are enriched in light rare earth elements, relative to MORBs, but whereas the alkali basalts exhibit uniformly steep patterns, the tholeiitic samples show much flatter profiles between La and Sm. The tholeiite trace element patterns are similar in shape to those for Hawaiian tholeiites^{23,24}, with normalized La $\approx$ normalized Ce, but abundances are higher in Ua Pou tholeiites. Tholeiites and alkali basalts at Ua Pou can also be distinguished by Zr/Nb (11.3 versus 4.8, average values, respectively), La/Nb (0.85 versus 0.98) and La/Ce (0.37 versus 0.51). In addition, on a spidergram diagram the tholeiites show significant depletions of Ba, Rb, Th, K and Sr relative to La.

To characterize further the two magma types present at Ua Pou, we have measured Sr, Nd and Pb isotopic compositions (Table 1). Previous isotopic studies of Marquesas Islands rocks^{11,12,25} have not recognized the importance of the tholeiites,

nor have they examined the compositional variation with age at a single island. Our results are in excellent agreement with the isotopic analyses of alkali basalts from Ua Pou reported by Vidal *et al.*¹². The data were obtained using separation techniques and mass spectrometric analysis, as described in refs 26 (Sr and Nd) and 27 (Pb). Measured Sr, Nd and Pb isotopic ratios are generally within the analytical uncertainty of initial ratios because of the young age of the samples. Corrections to $^{87}\text{Sr}/^{86}\text{Sr}$ were applied only to the high Rb/Sr phonolite samples. Our results are plotted on three diagrams: $^{143}\text{Nd}/^{144}\text{Nd}$ against $^{87}\text{Sr}/^{86}\text{Sr}$ (Fig. 2a), $^{87}\text{Sr}/^{86}\text{Sr}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ (Fig. 2b), and $^{207}\text{Pb}/^{204}\text{Pb}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ (Fig. 2c).

Tholeiites are distinguishable from alkali basalts and differentiated rocks using each of the three isotopic systems. The strontium composition of the tholeiites is among the least radiogenic reported from oceanic islands, while the alkali basalt $^{87}\text{Sr}/^{86}\text{Sr}$ values fall in the middle of the range for other Marquesas and Society Islands^{8,11,12}. The corresponding $^{143}\text{Nd}/^{144}\text{Nd}$ values are also distinctive ($\epsilon_{\text{Nd}} = 4.6$ –5.5 for tholeiites versus 0.6–2.1 for alkali basalts) and, together with the Sr data, form an array parallel to the Society Islands compositions but oblique to the mantle array^{5–8}. The extreme isotopic heterogeneity of the French Polynesian region has been noted, but at Ua Pou, as recognized for Nuku Hiva by Vidal *et al.*¹², nearly the entire range of variability exists on the scale of a single island. At Ua Pou the tholeiitic magmas have low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios and moderate $^{143}\text{Nd}/^{144}\text{Nd}$ ratios, indicative of a mantle source with low time-integrated Rb/Sr and a (chondrite-normalized) Nd/Sm < 1. The later alkali basalt magmas have high $^{87}\text{Sr}/^{86}\text{Sr}$ and low $^{143}\text{Nd}/^{144}\text{Nd}$, which would derive from a less depleted mantle source. This contrasts with the Hawaiian Islands volcanism, where the tholeiites have higher $^{87}\text{Sr}/^{86}\text{Sr}$ and lower ϵ_{Nd} values than the alkali basalts and post-erosional lavas^{9,10}.

The Pb compositions of the two magma types are equally distinct, as seen in the $^{207}\text{Pb}/^{204}\text{Pb}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ diagram (Fig. 2c). The tholeiites are most similar to rocks from Easter and Sala y Gomez Islands near the East Pacific Rise²⁵, whereas the alkali basalts lie in the field of other reported Pb isotopic analyses from the Society and Marquesas Islands^{12,25}. The Pb isotopic compositions of the Ua Pou tholeiites are not as extreme as those of rocks from Tubuai¹² (Austral Islands) which exhibit similar low $^{87}\text{Sr}/^{86}\text{Sr}$ and moderate ϵ_{Nd} . They are similar, however, to analysed rocks from Rimatara²⁸ and Rurutu²⁹, neighbouring volcanoes in the Austral Islands.

There has been much recent interest in the geographical distribution of isotopic compositions at oceanic islands, culminating in the Dupal anomaly proposal¹³, which postulates a region of higher $^{87}\text{Sr}/^{86}\text{Sr}$, $^{207}\text{Pb}/^{204}\text{Pb}$ and $^{208}\text{Pb}/^{204}\text{Pb}$, circling the globe at $\sim 30^\circ$ S. The new isotopic data for Ua Pou presented

here do not, however, support this view. The Marquesas Islands lie within the proposed Dupal anomaly belt, but only the alkali basalts have the appropriate radiogenic compositions. Apparently both Dupal and non-Dupal mantle reservoirs have been tapped at Ua Pou. The same sequence might also be seen at other French Polynesian islands, if the suspected early tholeiite phase of volcanism could be sampled. Hence, the regional significance of the Dupal anomaly, at least in this area, is questionable (see also refs 8, 26, 30).

It is evident from trace element and isotopic compositions that the tholeiites and alkali basalts at Ua Pou cannot be related by variable degrees of partial melting or crystal fractionation of primary melts from a common mantle or lithospheric source. Thus at least two, and probably three distinct source end-member compositions have contributed to volcanism at this island. In addition, the alkali basalts postdate the tholeiites in a clear age progression implying that the chemically distinct sources were tapped at different times. This suggests that the sources are not ubiquitous and continuously available for melting, but are likely to be physically separate. We will consider two possibilities for the spatial distribution of these source components: first, partial melting of both the mantle plume and the overlying lithosphere, and second, melting of the plume alone. In the first case the lithospheric mantle and plume have differing isotopic compositions and are the sources for the tholeiitic and alkali basalts respectively; in the second, both the alkalic and tholeiitic melts are derived from the plume, implying an isotopically heterogeneous plume.

The lower oceanic lithosphere probably consists of an unmelted mix of enriched mantle blobs and depleted asthenosphere which could possess considerable isotopic heterogeneity^{26,31-33} (Fig. 3a). Samples from the East Pacific Rise exhibit a rather small range of $^{87}\text{Sr}/^{86}\text{Sr}$ and ϵ_{Nd} compositions (Fig. 2a), but are quite variable in $^{206}\text{Pb}/^{204}\text{Pb}$ composition^{25,33} (Fig. 2b). Variation in these MORB compositions can be explained by mixing melts from depleted upper mantle with those from a small proportion of enriched mantle of the Rurutu/Tubuai type (low $^{87}\text{Sr}/^{86}\text{Sr}$, moderate ϵ_{Nd} , high $^{206}\text{Pb}/^{204}\text{Pb}$). Interestingly, rocks from Easter and Sala y Gomez Islands have compositions at the high- $^{206}\text{Pb}/^{204}\text{Pb}$ end of this trend²⁵ (Fig. 2b). Mantle upwelling in this region of the East Pacific Rise may be a major source for the enriched mantle blobs which became incorporated into the lower oceanic lithosphere by thickening of the plate during cooling away from the spreading centre.

At Ua Pou we propose that such heterogeneous oceanic lithosphere of 50–60 Myr age approached and crossed the Marquesas hotspot (Fig. 3a). Melts and heat from the mantle plume entered the lower lithosphere, where wall rock reached temperatures sufficient to melt. The plume and lithosphere melts could then mix to produce the magmas seen at the island. The trace element and isotopic compositions of the mixed magmas would vary depending on the degree of melting of each source and the proportions of melt contributed. At Ua Pou the isotopic composition of the early tholeiitic phase lies on a mixing line between MORB and Rurutu/Tubuai compositions, and reflects the proposed composition of the lower oceanic lithosphere beneath the island. Hence, in this model, melting of the lower lithosphere was significant and dominates the composition of tholeiitic magmas.

As the volcano migrated away from the hotspot, temperatures at the base of the lithosphere decreased. Smaller degrees of melting (of alkali basalt character) developed in the margin of the plume (Fig. 3a). These melts passed through the same column of oceanic lithosphere which had earlier yielded its low-temperature melting fraction; hence, the wall rock was much more refractory and less assimilation occurred at this stage, so that the alkali basalt compositions essentially reflect the isotopic composition of the plume. Contemporaneous with this phase of Ua Pou volcanism was tholeiitic, shield-building volcanism at Hiva Oa, the next major volcano upstream, and perhaps the

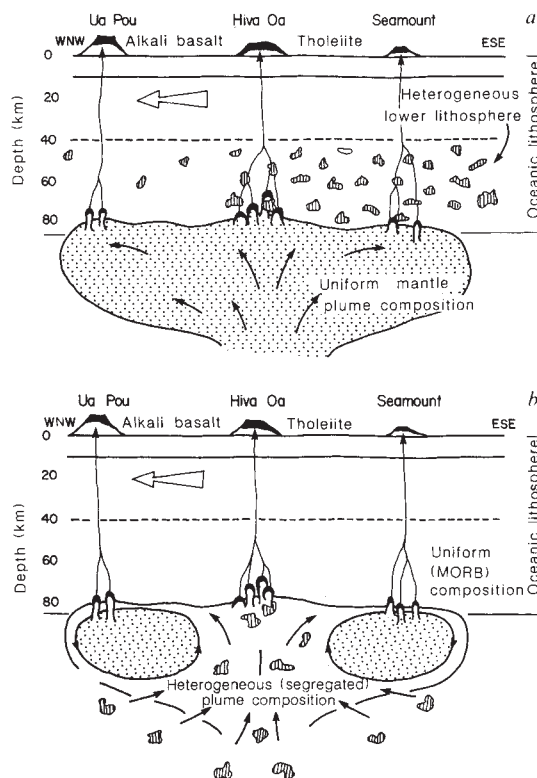


Fig. 3 Two models for the isotopically distinct magmas at Ua Pou, illustrated by vertical cross-sections along the line of the Marquesas Islands. Both models show volcanic activity at ~2.5 Myr, when alkali basalts erupted onto an older tholeiite shield at Ua Pou, concurrently with tholeiitic basalts at Hiva Oa and, possibly, with the initial seamount basalts beneath Fatu Hiva. *a*, Heterogeneous lower lithosphere. The plume (~400 km diameter) has a uniform, undepleted isotopic composition and is crossed by a Pacific plate composed of an upper section of uniform, MORB-like isotopic composition formed at the East Pacific Rise and an isotopically heterogeneous lower lithosphere accreted to the plate as it cooled away from the spreading ridge. Tholeiitic melts are formed over the centre of the plume as melts from the plume mix with melts from the lower-lithosphere wall rock. Alkali basalt melts erupt from the margin of the plume, where temperatures are cooler, and pass through previously heated lithosphere without significant contamination. *b*, Heterogeneous plume. The lithosphere has a uniform, MORB-like isotopic composition throughout and passes over a plume which has developed large-scale chemical heterogeneities by entraining depleted upper mantle material during its diapiric rise³⁴. The original plume material concentrates in a torus around the plume margin (shaded), while entrained material flows into the centre³⁵. Hence tholeiitic melts from the plume centre are MORB-like in isotopic character, whereas alkali basalt melts from the margin are more like the original plume. The lower lithosphere may melt but the isotopic differences in erupted magmas are controlled by heterogeneities in the plume.

seamount phase at Fatu Hiva, another 100 km to the south-east. From this model we would expect the same correlation of trace element and isotopic composition with time to emerge at other Marquesas islands.

An analogous model for the origin of Hawaiian tholeiitic and alkali basalts has been proposed by Chen and Frey⁹. At Hawaii, however, there is an inverse correlation between $^{87}\text{Sr}/^{86}\text{Sr}$ and abundances of incompatible elements; that is, the tholeiitic phase is dominated by the composition of melts from the plume, whereas the alkali basalts are thought to reflect the composition of the lower lithosphere. This relationship is thought to result from small degrees of melting of the lower lithosphere and variable proportions of mixing with plume-derived melts⁹. Although lithosphere melting occurs during the tholeiitic phase,

the volume of melts from the plume is sufficiently high that these determine the composition of Hawaiian tholeiites. During the formation of alkali basalt melts, however, very small (<1%) degrees of melting of the wall rock produce high concentrations of trace elements which dominate the composition of small-volume plume melts. Thus the isotopic composition of the Hawaiian alkali basalts reflects that of the lower lithosphere.

The source end-members for melting and magma mixing are different for the two island chains. At Ua Pou the enriched mantle, or plume composition, has $^{87}\text{Sr}/^{86}\text{Sr} > 0.7053$ and $\epsilon_{\text{Nd}} < 0.5$ and lies to the right of the Sr–Nd mantle array. This may be similar to, but possibly not so extreme as, the plume component for Society Islands alkali basalt magmas^{1,12}. The lower lithosphere beneath Hawaii, as reflected by the isotopic composition of the alkali basalts⁹, does not appear to contain the Rurutu/Tubuait component seen in the Ua Pou tholeiites. This model therefore requires that the oceanic lithosphere of the Pacific plate has accreted mantle blobs of different isotopic character.

An alternative explanation for the isotopically distinct phases of tholeiitic and alkalic volcanism at Ua Pou is that heterogeneities occur within the plume or diapir itself. These heterogeneities may be either a consequence of initial heterogeneities in the plume source, or result from the incorporation of asthenospheric mantle material into the diapir during its ascent. Recent experimental and theoretical studies^{34,35} have demonstrated that thermally activated diapirs will entrain a significant quantity of surrounding mantle material during their ascent. This is illustrated in Fig. 3b, where the central portion of the diapir consists of entrained depleted upper mantle (MORB-like plus mantle blobs), while the original plume material is contained in an outer torus. Tholeiitic melts are generated over the centre of the plume while smaller-volume alkali basaltic melts form over the perimeter. If isotopic heterogeneities are distributed uniformly through the diapir, then isotopically distinct magmas could be formed by variable degrees of partial melting. Larger degrees of melting (tholeiite) would tend to homogenize the variable composition of the diapir, whereas smaller degrees of melting (alkali basalt) would emphasize the composition of incompatible-element rich segregations^{31,32}.

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Geophysical evidence for the East Antarctic plate boundary in the Weddell Sea

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An improved Gondwanaland reconstruction compatible with geological and geophysical information from the surrounding oceans and continents seems to require microplates to solve the enigmatic pre-early-Mesozoic tectonic relation between West and East Antarctica¹. New multi-channel seismic reflection data from the southeastern Weddell Sea acquired during the 1984–85 Norwegian Antarctic Research Expedition (NARE) have outlined a linear WSW–ENE-trending basement ridge buried below the continental slope over a distance of 700 km. This structural high truncates the trend of the large sedimentary basins below the Filchner and Ronne ice shelves and may continue to within a few hundred kilometres of the Antarctic Peninsula. We interpret the basement ridge as part of the East Antarctic plate boundary during the break-up of Gondwana. The morphology and structure of this boundary show greater apparent similarity to a rifted or obliquely rifted margin than to the sheared margin which is predicted by current reconstructions^{2,3}. A linear East Antarctic plate margin extending to the vicinity of the Antarctic Peninsula makes any post-rift microplate motion in the Weddell Embayment unlikely.

Major linear basement highs have been found below the lower continental slope of the southeastern Weddell Sea margin (Fig. 1). In the north-east, the Explora Escarpment⁴ is characterized by a well-defined 10–20-km-wide basement ridge of 1–2 km elevation with respect to hyperbolated acoustic basement on the seaward side (Fig. 2). On the landward side, a wedge of seaward-dipping seismic reflectors, the Explora wedge, unconformably overlain by a 1–2-km-thick section of nearly flat-lying seismic sequences, abuts the escarpment. Towards the south-west (20°W), the Explora Escarpment becomes more subdued. The new NARE seismic data demonstrate that a major basement structure collinear with the Explora Escarpment extends at least 700 km farther towards the south-west, but also reveal considerable lateral variation in basement morphology (Figs 1, 2). Between 19 and 23°W a sharp, piece-wise-continuous reflector forms the upper boundary of a sequence of low-frequency seismic reflections whose landward termination is abrupt in some places and more diffuse in others (Fig. 3). We interpret these seismic reflection events as representing a region of seaward-dipping lava flows. We note that the top of this sequence is at a level comparable to the top of the escarpments to the north-east and west.

Further west, between 23 and 25°W, is a zone where acoustic basement forms a steep, landward-facing escarpment with down-faulted blocks of lava flows (?) at its base (Fig. 2, line 22–85). Here the escarpment appears to be offset from the landward boundary of the lava pile to the north-east.

At 26°W, the WSW-trending collinear basement structure is a subdued, 15–30-km-wide ridge with a relief of ~600 m which increases westwards to >2,000 m at 32°W (Fig. 2). We name the basement structure between 23 and 38°W the Andenes

Fig. 1 Locations of multi-channel seismic lines in the southeastern Weddell Sea acquired by Norwegian Antarctic Research Expeditions (NARE) in 1979 and 1985. Mapped escarpments and basement ridges (diagonal ruling) and regions of seaward-dipping lava flows (diagonal dashes) are shown. Bathymetry in metres from NARE data. Position of ice shelf (dashed line) and grounding line (solid line) from ref. 21.

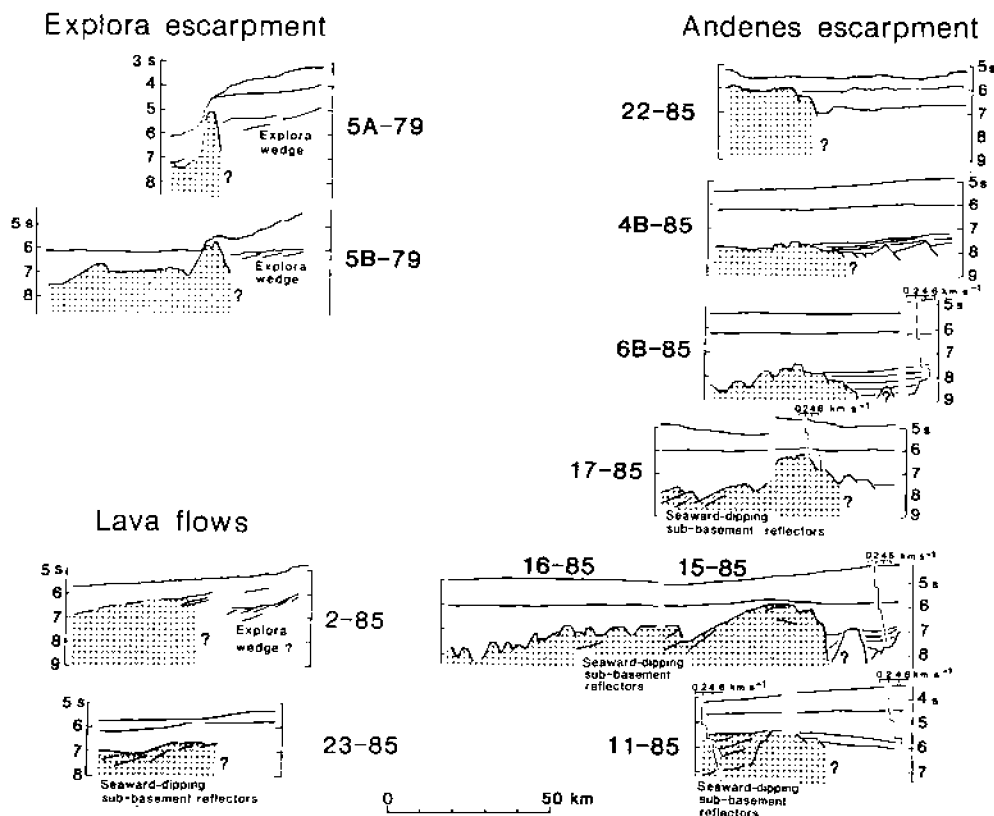
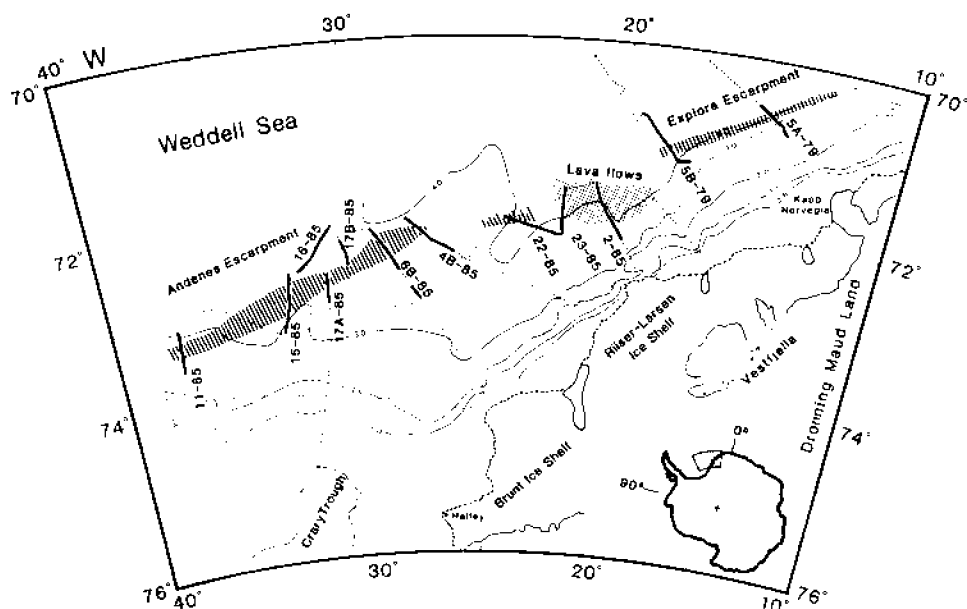


Fig. 2 Line drawings of seismic profiles located by heavy lines in Fig. 1. The interpreted extent of oceanic crust is indicated by V-pattern.

Escarpment after the expedition vessel *Andenes* of the Norwegian Coast Guard.

The Andenes and Explora Escarpments form a linear basement structure >1,000 km long which coincides with the transition between regions of contrasting reflection characteristics of acoustic basement (or the deepest seismic reflectors) and regional differences in magnetic field variations.

On the seaward side of the basement ridge is an uneven acoustic basement with abundant diffraction hyperbolae, with strong similarity to the acoustic character of oceanic crust (Fig. 2, lines 5B-79, 16-85 and 17-85). There is also evidence of seaward-dipping, low-frequency, sub-basement reflectors developed seaward of the escarpments (Fig. 2, lines 11-85, 16-85, 17-85 and 23-85).

On the landward side, the Explora wedge of seaward-dipping and divergent reflectors constitutes the deepest seismic sequence landward of the Explora Escarpment, and acoustic basement is observed only on isolated highs⁴. However, west of 23°W, we can observe acoustic basement for >150 km landward of the Andenes Escarpment and interpret it as a series of tilted blocks, locally with internal reflectors. Another characteristic feature in this area is the distinct low-frequency acoustic character and undisturbed nature of the material ($v_p > 4.5 \text{ km s}^{-1}$) infilling topographic lows in acoustic basement (Fig. 2, lines 4B-85, 6B-85 and 15-85).

The magnetic signature associated with the Explora Escarpment is a positive magnetic anomaly, whereas the relationship along the Andenes Escarpment is more complex (Fig. 4). On

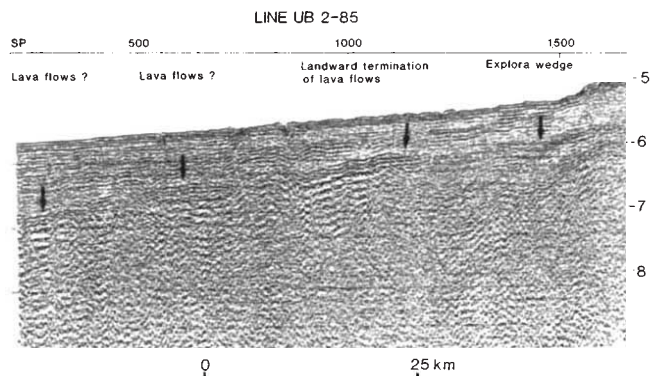


Fig. 3 Seismic reflectors interpreted as seaward-dipping lava flows. SP, shot-point.

the landward side of the escarpments, and paralleling the margin outside the Riiser-Larsen Ice Shelf, is a positive-negative pair of long-wavelength (<100 km) magnetic anomalies which extend southwestward towards the Filchner Ice Shelf and diverge with the trend of the basement ridge^{5,6}. To the south, short-wavelength, high-amplitude magnetic anomalies are associated with magnetic⁵ and acoustic⁷ basement of the east Antarctic craton exposed at the sea floor in a 50–70-km-wide swath along the barrier between Filchner Ice Shelf and Halley (30–37° W). In the northern part of the Soviet survey area (74° S), there is a conspicuous change in magnetic field character towards short-wavelength, high-amplitude, E–W-trending lineations collinear with the Andenes Escarpment. This strongly suggests that the tectonic grain represented by the escarpments may continue

towards the Antarctic Peninsula at least to 55° W.

To the north of the Andenes–Explora escarpments, lineated magnetic anomalies have been related to oceanic crust formed by seafloor spreading^{6,8}. Thus, the escarpments could represent an ancient Gondwana plate boundary in the Weddell Embayment, between oceanic crust to the north and crust of unknown origin to the south.

Gondwana reconstructions using current knowledge of magnetic anomalies in the South Atlantic and Indian Ocean predict large initial strike-slip motion along the Dronning Maud Land (DML) margin during the early opening^{2,3}. The lineated magnetic anomalies observed in the Weddell Sea are strong evidence of underlying oceanic crust formed at a spreading centre sub-parallel to the Explora escarpment^{6,8}. Thus the Andenes–Explora escarpments may have one or several modes of origin: (1) a fracture zone; (2) a rifted margin; (3) a sheared margin subsequently overprinted by rifting.

The morphological signature of a fracture zone in the oceanic crustal domain is most often a paired basement ridge and trough⁹, whereas a single ridge is most often observed at sheared continental margins^{10,11}. Also, in the latter environment, the ancient fracture zone location at the margin is associated with a precipitous increase in water depth from a shelf or plateau to abyssal depths.

Basement morphology and structure along the Explora Escarpment have affinities with those of a sheared continental margin (Fig. 2). However, the collinear region of lava flows between 18 and 23° W and the Andenes Escarpment shows stronger similarities to a rifted margin segment, the principal evidence being the seaward-dipping sub-basement reflectors to the north of the lava front (line 23-85) or escarpment (lines 16-85, 17-85), respectively. Wedges of seaward-dipping sub-

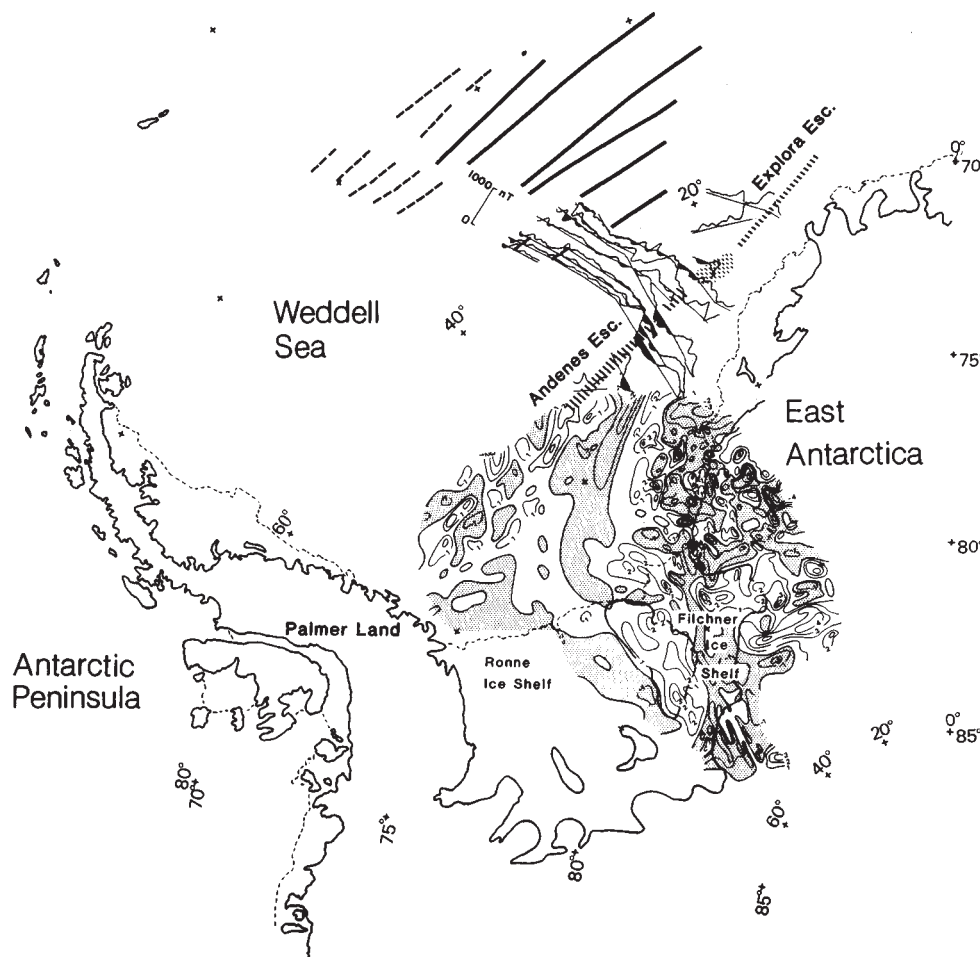


Fig. 4 Compilation of aeromagnetic data⁵ (areas of negative anomalies shaded) and marine magnetic data⁶, together with mapped magnetic lineations in the Weddell Embayment.

basement reflectors, often terminated on the landward side by an escarpment, have been observed along several rifted continental margins and are thought to be at or near the transition from continental to oceanic crust¹². Paralleling the Andenes and Explora escarpments at a distance of <300 km towards the south is the 130-km-long Vestfjella chain of nunataks, which experienced a regime of NW-SE tension during the early to middle Jurassic¹³⁻¹⁵. A 1-2-km-thick section of mostly tholeiitic basalts was laid down in subaerial conditions. The volcanic pile is cut by many dykes of predominantly NE-SW strike, and by normal faults with no significant component of strike-slip. Also, basement depths landward of the escarpments (Fig. 2) require a thin crust from isostatic considerations. Thus, the evidence available favours an interpretation of the Andenes-Explora escarpments as structures of a rifted margin or possibly a plate boundary with oblique spreading rather than pure strike-slip. A sequence of events beginning with a large initial shear and subsequent overprint by rifting cannot be ruled out, but is considered less likely in view of the structural history of adjacent land areas, as well as the structural simplicity of the Andenes and Explora escarpments.

A major event in the evolution of the Weddell Embayment was mid-Jurassic crustal extension between the East Antarctic craton and the Pacific facing arc¹⁶, forming major north-south grabens below the Ronne and Filchner ice shelves^{5,17}. A Gondwana plate margin represented by the linear Andenes-Explora escarpments and their western continuation cuts across the early extensional tectonic trend (Fig. 4), indicating a post-mid-Jurassic change in the regional stress field.

A post-rift-phase Filchner microplate^{1,3} probably did not exist. The available multi-channel seismic data^{4,7} (Fig. 1) demonstrate that the sediments below the continental slope and shelf between 15 and 40° W are characterized by a total absence of fold and fault structures induced by post-rift basement tectonics. Also, preliminary results of a seismic survey by *Polarstern*¹⁸ along portions of a traverse from the East Antarctic craton to the Antarctic Peninsula, outside the Filchner and Ronne ice shelves, show the upper 1 s (two-way travel time) of sediments to be undisturbed except in an area within 150 km of the peninsula. Thus, the locus of any post-rift relative motion between the Antarctic Peninsula^{19,20} and the East Antarctic craton is certainly west of 40° W and probably near the peninsula itself.

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Capacity of purified Lyt-2⁺ T cells to mount primary proliferative and cytotoxic responses to Ia⁻ tumour cells

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Allogeneic gene products of the major histocompatibility complex, the *HLA* complex in man and the *H-2* complex in mice, induce T lymphocytes to exert powerful mixed lymphocyte reactions (MLR) and cell-mediated lympholysis (CML). In mice, the subset of T cells carrying the L3T4 surface antigen but lacking the Lyt-2 antigen responds predominantly to H-2 class II (Ia) differences whereas the L3T4⁻ Lyt-2⁺ subset reacts to class I (K/D) differences^{1,2}. For primary responses the stimulus for MLR and CML appears to be controlled by Ia⁺ cells of the macrophage/dendritic cell lineages, for both L3T4⁺ and Lyt-2⁺ cells³⁻⁶. The finding that Ia⁺ cells are required for responses involving Lyt-2⁺ cells has been taken to imply that triggering of these cells is controlled by Ia-restricted L3T4⁺ cells^{7,8}. Lyt-2⁺ cells have thus come to be regarded as crippled cells which are heavily dependent on 'help' from other T cells⁹⁻¹¹. This well-entrenched view is challenged by evidence presented here that purified Lyt-2⁺ cells can give high primary responses to certain Ia⁻ tumour cells *in vitro*.

Recent studies from this laboratory showed that highly purified Lyt-2⁺ cells were able to mount high primary MLR and CML responses to class I but not class II H-2 differences *in*

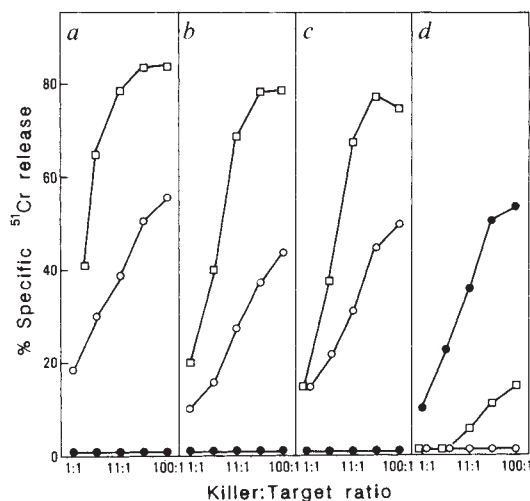


Fig. 1 Cytolytic activity of purified B6 (H-2^b) Lyt-2⁺ cells cultured for 4 days *in vitro* with P815 (H-2^d) tumour cells. Doses of 2×10^6 responder cells were cultured with lightly irradiated (1,500 rad) DBA/2 (H-2^d) spleen cells or with 0.5×10^6 heavily irradiated (20,000 rad) P815 tumour cells in a volume of 2 ml in 24-well plates; as controls for specificity of lysis, DBA/2 T (J11d-treated LN) cells were cultured with B10 (H-2^b) spleen cells. In experiment c, the purified Lyt-2⁺ cells were pretreated with anti-Ia^b antibody plus C' before culture. After 4 days the cells pooled from several wells were counted and then cultured in varying numbers for 3 h at 37 °C with fixed numbers (10^4) of ⁵¹Cr-labelled target cells, that is, P815 cells (□), concanavalin A-stimulated B10.D2 (H-2^d) (○) or B10 (H-2^b) (●) spleen cells; target cells were labelled with 300 μCi ⁵¹Cr per 4×10^6 cells at 37 °C for 1 h and then washed thoroughly. The per cent ⁵¹Cr release from target cells was measured by standard techniques, taking release of isotope from detergent-treated cells as 100% release. Each data point represents the mean per cent specific ⁵¹Cr release of triplicate cultures; the data in a, b, and in c, d were obtained in two different experiments.

Table 1 Primary MLR of B6 T cells and purified B6 Lyt-2⁺ cells stimulated with H-2-incompatible spleen cells or P815 tumour cells

Stimulators	No. of stimulators ($\times 10^5$)	³ H-TdR incorporation (c.p.m. $\times 10^3$) with responders (2×10^5)								
		B6 T (H-2 ^b)			B6 Lyt-2 ⁺ (H-2 ^b)			No responders		
		Day 3	Day 4	Day 5	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
B6 spleen (H-2 ^b)	0.2	0.3	0.4	1.3	0.1	0.1	0.1	—	—	—
	1	0.6	1.1	7.6	0.1	0.1	0.1	—	—	—
	5	2.3	6.7	17.8	0.3	0.7	0.7	0.2	0.3	0.5
DBA/2 spleen (H-2 ^d)	0.2	15.9	34.1	51.8	18.8	44.5	25.5	—	—	—
	1	86.7	171.4	44.6	82.4	188.3	16.1	—	—	—
	5	144.2	275.0	58.9	132.6	263.0	44.9	0.4	0.5	0.5
P815 (H-2 ^d)	0.2	28.0	62.7	14.4	38.8	66.7	11.5	0.4	0.5	0.2
	1	8.2	16.9	16.3	26.8	49.4	30.6	0.4	0.3	0.2
	5	4.3	3.6	3.8	3.3	3.6	4.4	2.3	2.7	2.1

T cells and T-cell subsets were purified from pooled lymph nodes (LN) of adult B6 mice as described elsewhere². To purify T cells, LN cells were pretreated at 37 °C for 1 h *in vitro* with an anti-B-cell monoclonal antibody (J11d) plus guinea pig serum as a source of complement (C'); the surviving cells (>98% Thy 1⁺) were then passed through Ficoll gradients to remove dead cells. To prepare Lyt-2⁺ cells, LN cells were first pretreated at 37 °C *in vitro* with a mixture of J11d and anti-L3T4 (GK1.5) antibodies plus C'. The surviving cells were then washed and allowed to adhere to anti-Lyt-2-coated dishes for 1 h at 4 °C. After gently washing non-adherent cells from the dishes, the adherent cells were eluted by vigorous pipetting. The adherent cells were >99% Lyt-2⁺ by FACS analysis and contained no detectable L3T4⁺ cells². For MLR, doses of 2×10^5 B6 T or Lyt-2⁺ responder cells were cultured in flat-bottom microtitre plates with varying numbers of lightly irradiated (1,500 rad) normal B6 or DBA/2 spleen cells or with heavily irradiated (20,000 rad) *in vitro*-passaged P815 tumour cells in a final volume of 200 μ l. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and standard additives²; IL-2 was not added to the cultures. Cultures were pulsed with 1 μ Ci tritiated thymidine (³H-TdR) 18 h before collection. The data show the mean levels of radioactivity in triplicate cultures. s.d., omitted for simplicity, were generally within 10–20% of the mean. Pretreatment of P815 cells with mitomycin C rather than irradiation led to comparable MLR with B6 Lyt-2⁺ cells.

*vitro*²; other workers have reported similar findings¹². MLR to class I differences were not reduced by removing T cells from the stimulator population or by adding anti-L3T4 monoclonal antibody to the cultures^{2,12}; addition of anti-Ia monoclonal antibody caused only minimal inhibition of the response. Although these findings suggested that the response of Lyt-2⁺ cells could not be attributed to minor contamination of the cultures with L3T4⁺ cells, removal of Ia⁺ cells from the stimulator population (spleen cells) virtually abolished the response^{2,12}. At face value the simplest explanation of this finding is that the response of Lyt-2⁺ cells to alloreactive class I molecules requires co-recognition of class II (Ia) molecules. The problem with this notion is that, unless one invokes cryptic participation of L3T4⁺ cells, it is difficult to envisage how Ia molecules might control Lyt-2⁺ cell induction. In addition, one must account for the fact that certain Ia⁺ cells—small B lymphocytes—are non-stimulatory for unprimed Lyt-2⁺ cells⁶. An alternative possibility is that Ia⁺ cells with stimulatory function display a putative 'second signal' required by Lyt-2⁺ cells^{12,13}, with the joint expression of Ia molecules and the second signal being largely coincidental. This raises the question of whether some Ia⁺ cells might be stimulatory for Lyt-2⁺ cells. Rammensee *et al.*¹⁴ have shown that Ia⁺ Lyt-2⁺ T cells have the capacity to 'veto' the induction of allogeneic Lyt-2⁺ cytolytic precursors, the veto function of T cells being attributed to the failure of these cells to express a requisite second signal. Since spleen cell suspensions depleted of Ia⁺ cells consist largely of T cells, the poor antigen-presenting cell function of Ia⁺ spleen cells thus does not preclude the possibility that a spectrum of cell types might act as antigen-presenting cells for Lyt-2⁺ cells, the notable exception being T cells. To assess this idea, we arbitrarily tested the antigen-presenting function of Ia⁺ tumour cells.

Although certain Ia⁺ tumours, such as the P815 mastocytoma, are known to elicit primary T-cell responses *in vitro*^{13,15}, it is unclear whether these responses require help from the L3T4⁺ T-cell subset. Table 1 compares the capacity of B6 (H-2^b) T cells and >99% purified B6 Lyt-2⁺ cells to mount primary MLR to DBA/2 (H-2^d) spleen stimulators (1,500 rad) or P815 (H-2^d) tumour stimulators (20,000 rad) in the absence of added interleukin-2 (IL-2); the P815 tumour, of DBA/2 origin, completely lacks Ia molecules as assessed by fluorescence-activated cell sorter (FACS) analysis, but is strongly positive for class I

molecules (data not shown). B6 T and B6 Lyt-2⁺ cells both gave high responses to DBA/2 spleen. Peak responses occurred on day 4 and the responses decreased progressively as the dose of stimulators was lowered. With P815 stimulators, large doses of tumour cells (5×10^5 per culture) elicited virtually no MLR. Low tumour doses (10^4 – 10^5), by contrast, elicited highly significant MLR. Responsiveness to the P815 tumour seemed to be restricted to Lyt-2⁺ cells because: (1) responses were appreciably higher with B6 Lyt-2⁺ cells than with unseparated B6 T cells (Table 1), (2) there was no response to the tumour using purified B6 L3T4⁺ cells (Table 2, expts a, b) (implying that the tumour cells remained Ia⁺ in culture), and (3) in marked contrast to anti-Lyt-2 monoclonal antibody, adding anti-L3T4 to the cultures failed to inhibit the response of B6 Lyt-2⁺ cells to the tumour (Table 2, expt a; the response to the bm1 and bm12 mutants controlled for the specificity of the blocking effects of anti-Lyt-2 and anti-L3T4 monoclonal antibodies, see Table 2 legend). Heat-killed P815 cells and P815 cells exposed to ultraviolet light were completely non-stimulatory, even when reconstituted with recombinant IL-1 (data not shown).

The response of B6 Lyt-2⁺ cells to the P815 tumour appeared to be specific for H-2 antigens, as the tumour failed to stimulate H-2-identical DBA/2 Lyt-2⁺ cells (Table 2, expt b) but did stimulate Lyt-2⁺ cells from another H-2^b strain, C3H.SW (Table 2, expt a). Further evidence for antigen specificity was obtained by studying the capacity of the tumour to elicit CML activity. In all three experiments performed, two of which are illustrated in Fig. 1, culturing B6 Lyt-2⁺ cells for 4 days with irradiated P815 cells in the absence of added IL-2 led to high levels of lysis against ⁵¹Cr-labelled P815 cells. With concanavalin A-stimulated spleen blast cells as targets, lysis was high on B10.D2 (H-2^d) targets but absent on B10 (H-2^b) targets, implying that lysis was directed to H-2^d determinants rather than to tumour-specific antigens. The specific lytic activity of B6 Lyt-2⁺ cells cultured with P815 stimulators was only slightly lower than with cells cultured with DBA/2 spleen stimulators (compare Fig. 1a and b). Pretreating the B6 Lyt-2⁺ cells with anti-I-A^b antibody plus complement (C') to remove any residual Ia⁺ cells before culture failed to impair CML activity (Fig. 1c).

The above results indicate that Ia⁺ P815 tumour cells are able to stimulate purified allogeneic Lyt-2⁺ cells to proliferate extensively and differentiate into H-2-specific (presumably class I-

Table 2 MLR of T-cell subsets to P815 (H-2^d) and L929 (H-2^k) tumour cells: failure of anti-L3T4 antibody to inhibit MLR of Lyt-2⁺ cells

Stimulators	No. of stimulators ($\times 10^5$)	No. of responders ($\times 10^5$)	Antibody added to cultures	³ H-TdR incorporation (c.p.m. $\times 10^3$) with responders			
				B6 Lyt-2 ⁺ (<i>bbbb</i>)	C3H.SW Lyt-2 ⁺ (<i>bbbb</i>)	B6 L3T4 ⁺ (<i>bbbb</i>)	No responders
Expt <i>a</i>							
B6 spl (<i>bbbb</i>)*	5	1	—	0.3	0.8	3.1	—
	5	2	—	0.8	1.2	6.3	—
bml spl (<i>bmlbbb</i>)	5	1	—	74.5	57.4	3.8	—
	5	1	Anti-L3T4	74.5	51.0	—	—
	5	1	Anti-Lyt-2	2.7	2.7	—	—
	5	2	—	178.3	82.0	—	—
bm12 spl (<i>bbm12bb</i>)	5	1	—	2.2	1.9	22.9	—
	5	1	Anti-L3T4	—	—	2.1	—
	5	1	Anti-Lyt-2	—	—	30.3	—
	5	2	—	—	—	81.5	—
P815 (<i>dddd</i>)	0.5	2	—	25.4	18.2	0.3	0.2
	0.5	2	Anti-L3T4	21.9	17.6	—	—
	0.5	2	Anti-Lyt-2	0.8	0.6	—	—
				B6 Lyt-2 ⁺ (<i>bbbb</i>)	DBA/2 Lyt-2 ⁺ (<i>dddd</i>)	B6 L3T4 ⁺ (<i>bbbb</i>)	No responders
Expt <i>b</i>							
B6 spl (<i>bbbb</i>)	5	2	—	1.1	29.0	4.2	0.1
DBA/2 spl (<i>dddd</i>)	5	2	—	104.1	0.4	210.2	0.1
P815 (<i>dddd</i>)	0.25	2	—	32.3	0.5	1.1	0.1
	0.5	2	—	40.9	1.2	0.8	0.1
	1	2	—	43.0	1.4	3.1	0.2
				B6 Lyt-2 ⁺ (<i>bbbb</i>)	CBA/Ca Lyt-2 ⁺ (<i>kkkk</i>)	No responders	
Expt <i>c</i> [†]							
B6 spl (<i>bbbb</i>)	5	1	—	0.2	20.7	0.1	
CBA/Ca spl (<i>kkkk</i>)	5	1	—	55.5	0.7	0.3	
bml spl (<i>bmlbbb</i>)	0.2	1	—	2.2	0.8	0.3	
	5	1	—	70.5	31.1	0.1	
	5	1	Anti-L3T4	62.0	—	—	
	5	1	Anti-Lyt-2	3.2	—	—	
L929 (<i>kkkk</i>)	0.04	1	—	5.5	0.6	0.5	
	0.2	1	—	16.7	1.1	1.1	
	0.2	1	Anti-L3T4	24.8	—	—	
	0.2	1	Anti-Lyt-2	2.2	—	—	
	1	1	—	22.4	1.7	1.7	
	5	1	—	0.6	0.4	0.4	

Lyt-2⁺ cells were prepared as for Table 1. An analogous procedure was used to prepare L3T4⁺ cells; that is, pretreatment of LN cells with J11d+anti-Lyt-2 (3.168) antibodies + C' followed by positive panning on dishes coated with anti-L3T4. The P815 mastocytoma and the L929-transformed fibroblast line were both totally I-A-negative by FACS analysis but were strongly positive for expression of class I molecules. As for P815, the L929 cells were passaged *in vitro* without feeder cells; the cells were exposed to 20,000 rad before use as stimulators. The amount of antibody added to the cultures was 2 μ l of undiluted ascites fluid for anti-Lyt-2 and 2 μ l of 1:10 diluted ascites fluid for anti-L3T4. As controls for the specificity of inhibition by anti-L3T4 and anti-Lyt-2, these antibodies were added to cultures in which MLR were directed solely to a class I H-2 difference (B6 Lyt-2⁺ cells responding to the H-2K-different B6.C-H-2^{bm1} (bm1) mutant) or to a class II H-2 difference (B6 L3T4⁺ cells responding to the I-A-different B6.C-H-2^{bm12} (bm12) mutant); as reported elsewhere² (confirmed in the table), anti-L3T4 selectively inhibits anti-class II MLR whereas anti-Lyt-2 selectively inhibits anti-class I MLR. MLR (mean of triplicate cultures) were measured on day 4 for each experiment shown.

* Alleles of H-2 subregions, that is, H-2K, I-A, I-E, H-2D.

† Responder T cells in this experiment were pretreated with anti-I-A^b + C' before culture.

specific) cytotoxic cells in the absence of exogenous IL-2 (although IL-2 production by a subset of Lyt-2⁺ T 'helper' cells⁶ cannot be excluded). Similar findings were observed with the L929 (H-2^k) Ia⁻ transformed fibroblast line (tested only for MLR) (Table 2, expt c). In the case of non-neoplastic cells, we have obtained preliminary evidence that Thy 1⁺ Ia⁻ cells from normal bone marrow can stimulate primary MLR by allogeneic Lyt-2⁺ cells (unpublished data). Thus, the capacity to stimulate Lyt-2⁺ cells is apparently not a property unique to Ia⁻ tumour cells.

In contrast to these findings with Thy 1⁺ cells, studies with three Thy 1⁺ Lyt-2⁻ T-cell tumours have shown that, like normal T cells, these tumours cannot stimulate unprimed allogeneic Lyt-2⁺ cells in the absence of added IL-2 (unpublished data of

the authors); the reason for this is unclear. Interestingly, the ability of T cells to mediate veto function is largely restricted to activated Lyt-2⁺ T-killer cells and is abolished by irradiation¹⁴. Hence, the poor stimulatory function of typical small T cells (for example, anti-Ia + C'-treated spleen) and Lyt-2⁻ T tumours is unlikely to reflect a veto effect. Moreover, we have seen no evidence of suppression when Lyt-2⁺ responders are exposed to a mixture of spleen stimulators supplemented with irradiated small T cells or T tumours. The most likely explanation for the poor stimulatory function of (non-cytotoxic) T cells is that these cells simply lack some requisite second signal required by Lyt-2⁺ cells.

Although the nature of the putative second signal provided by Thy 1⁺ Ia⁻ H-2-different tumour cells is unknown, it is

possible that such tumours are directly immunogenic for Lyt-2⁺ cells *in vivo*, involvement of L3T4⁺ cells responding to 'processed' tumour H-2 antigens being unnecessary for tumour rejection. In this respect, we now have preliminary evidence that the subcutaneous growth of P815 tumour cells in irradiated B6 mice can be prevented by mixing the injected tumour cells with unprimed purified B6 Lyt-2⁺ cells (unpublished data).

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Note added in proof: We have recently found that one T cell tumour (EL4) does stimulate high primary MLR by allogeneic Lyt-2⁺ cells.

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Increased levels of myelin basic protein transcripts gene in virus-induced demyelination

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In multiple sclerosis, a demyelinating disease of young adults, there is a paucity of myelin repair in the central nervous system (CNS) which is necessary for the restoration of fast saltatory conduction in axons^{1,2}. Consequently, this relapsing disease often causes marked disability. In similar diseases of small rodents, however, remyelination can be quite extensive, as in the demyelinating disease caused by the A59 strain of mouse hepatitis virus (MHV-A59)^{3,4}, a coronavirus of mice. To investigate when and where oligodendrocytes are first triggered to repair CNS myelin in such disease, we have used a complementary DNA probe specific for one major myelin protein gene, myelin basic protein (MBP), which hybridizes with the four forms of MBP messenger RNA in rodents⁵. Using Northern blot and *in situ* hybridization techniques, we previously found that MBP mRNA is first detected at about 5 days after birth, peaks at 18 days and progressively decreases to 25% of the peak levels in the adult⁵⁻⁷. We now report that in spinal cord sections of adult animals with active demyelination and inflammatory cells, *in situ* hybridization reveals a dramatic increase in probe binding to MBP-specific mRNA at 2-3 weeks after virus inoculation and before remyelination can be detected by morphological methods. This increase of MBP-specific mRNA is found at the edge of the demyelinating area and extends into surrounding areas of normal-appearing white matter. Thus, *in situ* hybridization with myelin-specific probes appears to be a useful method for detecting the timing, intensity and location of myelin protein gene reactivation preceding remyelination. This method could be used to elucidate whether such a reactivation occurs in multiple sclerosis brain tissue. Our results suggest that in mice, glial cells react to a demyelinating process with widespread MBP mRNA synthesis which may be triggered by a diffusible factor released in the demyelinated areas.

Earlier studies have shown that some MHV-A59 strains can cause chronic demyelination in mice and rats^{4,8-15}. For instance, a high incidence of demyelination occurs in C3H and C57 black mice^{3,4} after intracerebral inoculation of the prototype MHV-A59 strain¹⁶. The virus replicates preferentially in oligodendrocytes, destroying the cells in focal areas of the white matter during the first weeks of infection^{3,4,8-15}. Viral antigen persists in the cytoplasm of oligodendrocytes within the demyelinating

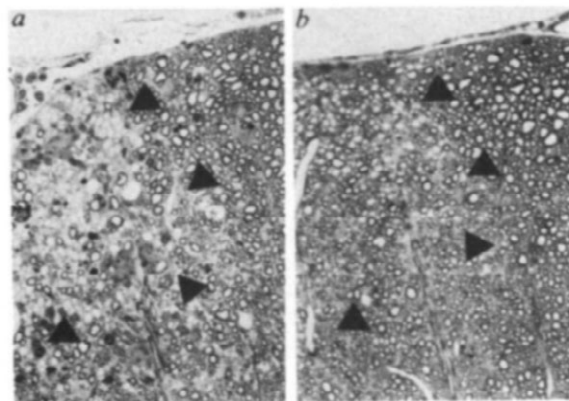


Fig. 1 Micrographs showing demyelinated (a) and remyelinated (b) areas (delineated by arrowheads) in the anterior column of mice at 4(a) and 10(b) weeks post-inoculation with MHV-A59. Active demyelination is seen at 4 weeks while thinly remyelinated axons are seen at 10 weeks. Animals were perfused with 4% glutaraldehyde in Sorensen phosphate buffer, and slices of various levels of the spinal cord were embedded in Epon. 1-µm-thick sections were stained with toluidine blue. ×200.

lesions for up to 4 weeks³. Untranslated viral genome may be present in other areas of the CNS and in other cell types¹⁷.

In the present investigation, 4-week-old C57Bl/6J mice (obtained from Jackson Laboratories, Bar Harbor, Maine) were injected intracerebrally with 500-1,000 plaque-forming units of MHV-A59 (obtained from Dr L. S. Sturman, New York State Department of Health, Albany, New York). At 1, 2, 3, 4 and 8 weeks after injection, groups of three or four mice were perfused through the heart with periodate-lysine-formaldehyde¹⁸, and the brains and spinal cords of the perfused animals were then dissected. Transversely cut slices of various regions of the brain and spinal cord were prepared by freezing and cryosectioning for *in situ* hybridization as described in Fig. 2 legend. As a probe, we used a small cDNA clone, NZ-112, which encodes amino acids 60-93 of mouse MBP⁵. By adding 20-25³⁵S-labelled dATP residues to the 3' ends of the gel-purified DNA fragments using terminal deoxynucleotidyl transferase, a specific activity of 1-2 × 10⁹ d.p.m. per µg DNA was obtained^{6,7}. Other CNS tissue slices were embedded in paraffin for histological examination and immunocytochemistry. For histology, paraffin sections were stained with Luxol fast blue and cresyl violet or haematoxylin/eosin. For immunocytochemistry, paraffin sections were incubated with dilutions of mouse or goat anti-MBP antibodies and stained by the peroxidase-antiperoxidase method¹⁹. In addition, two or three mice at each time point were perfused and processed as described in Fig. 1 legend in order to analyse the details of the demyelinating and remyelinating process in semi-thin plastic sections.

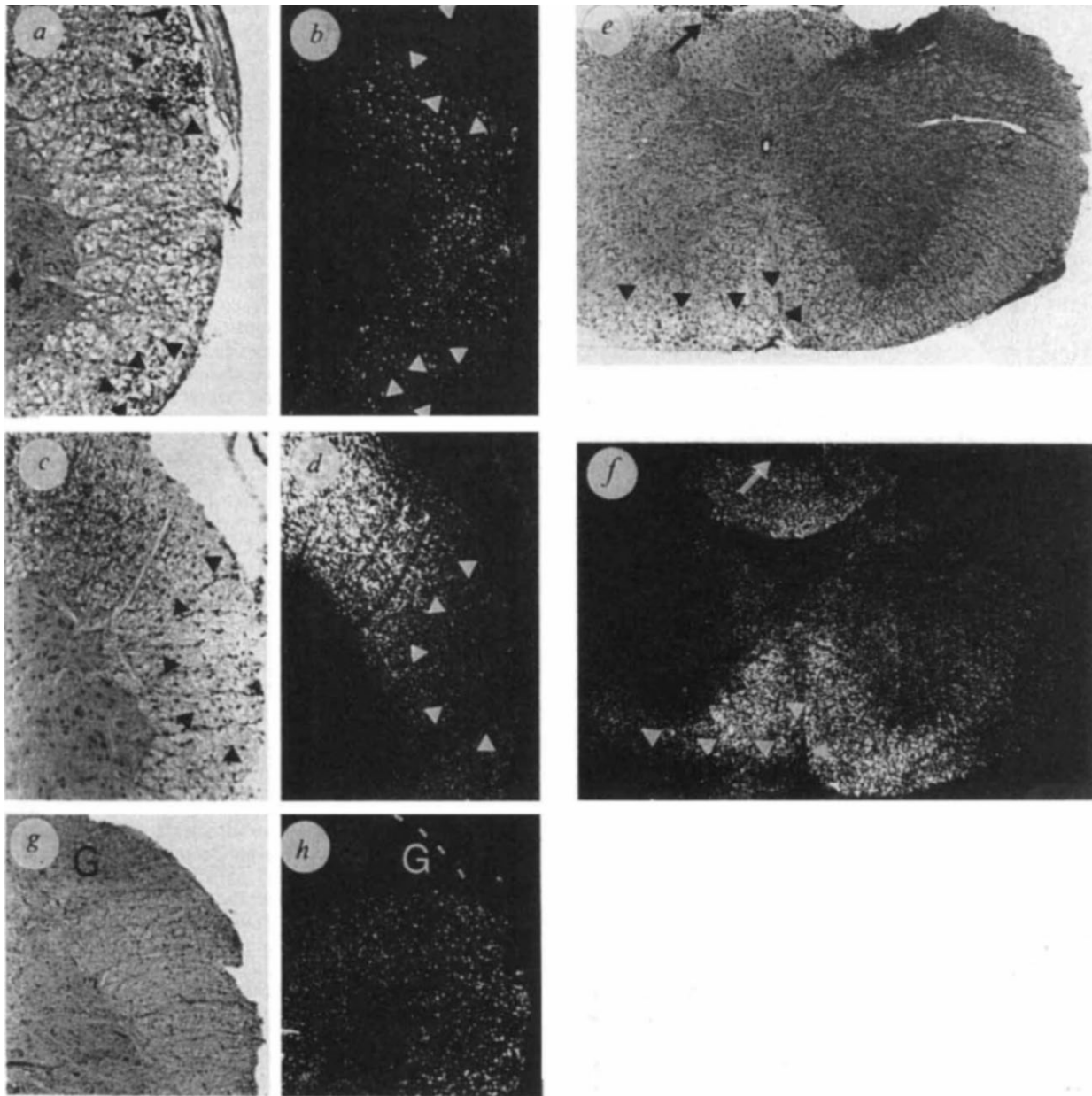


Fig. 2 Micrographs showing the location of MBP-specific transcripts in cervical spinal cords of virus-infected animals at 2(*a,b*), 3(*c,d*), 4(*e,f*) and 8(*g,h*) weeks post-inoculation. Bright-field views of histological stained sections are shown (*a,c,e,g*), together with corresponding dark-field cryostat sections after *in situ* hybridization (*b,d,f,h*). The number of grains observed in dark field is directly proportional to the amount of hybridization with the MBP probe and thus the number of MBP-specific transcripts. Note some hybridization of the MBP-specific probe in white matter at 2 weeks post-inoculation, with a decrease in labelling in the lesion containing inflammatory cells (delineated by arrowheads). In contrast, at 3 and 4 weeks, intense hybridization is seen in regions surrounding the lesions (delineated by arrowheads) which are devoid of label. Increased hybridization extends into the normal-looking white matter: in *f*, the left ventral column has a lesion (under arrowheads) but increased label is also seen in the right ventral column. The dorsal right column also has a small lesion (arrow) surrounded by increased label. At 8 weeks (*g,h*), there is still a slight increase in white matter labelling without the differential distribution (G, grey matter of substantia gelatinosa) observed in the 3–4-week samples. $\times 200$.

Methods. For these *in situ* hybridization experiments, samples from the spinal cord were cryoprotected in 15% sucrose (RNase-free) before freezing in liquid nitrogen. Cryostat sections ($10\ \mu\text{m}$ thick) were cut and hybridized as described in detail previously⁷. Briefly, the sections were treated with 0.2 M HCl for 20 min, washed in phosphate-buffered saline, incubated with $1\ \mu\text{g ml}^{-1}$ proteinase K (Boehringer Mannheim) for 15 min at 37°C , and then with the hybridization mixture without the probe for 2 h. The hybridization mixture was as follows: 50% formamide, $2\times\text{SSC}$, $1\times\text{Denhardt's solution}$, salmon sperm DNA (Sigma) at $450\ \mu\text{g ml}^{-1}$, *Escherichia coli* transfer RNA (Sigma) at $500\ \mu\text{g ml}^{-1}$, $200\ \mu\text{g ml}^{-1}$ poly(A) (Sigma), 40 nM oligonucleotide d(pT)₈ (Collaborative Research, Inc.). The probe, at a concentration of $100\ \mu\text{g ml}^{-1}$ and specific activity of 1×10^9 d.p.m. ml^{-1} , together with the salmon sperm DNA and *E. coli* tRNA, was denatured for 3 min at 100°C , quenched on ice for 3 min and mixed with the other constituents of the hybridization mixture with 40 mM dithiothreitol. The mixture was incubated for 15 min at 50°C , denatured again and incubated at 50°C for another 15 min. Each section was then incubated at 40°C with $10\ \mu\text{l}$ of the hybridization mixture in a sealed chamber overnight (about 16 h), washed in $0.1\times\text{SSC}$ at 55°C for 3 h, treated with 75% and 95% ethanol with 300 mM ammonium acetate and air-dried. The slides were dipped in a Kodak NTB-2 emulsion with 300 mM ammonium acetate and stored at 4°C for 3 days before being developed using standard procedures. The sections were counterstained with cresyl violet before mounting in Permount.

The clinical and pathological features of the disease have been described elsewhere^{3,4}. Briefly, 5–7 days after infection, the mice developed signs of acute disease characterized by apathy, hunched posture, ruffled fur and tremor. Some mice exhibited paresis spontaneously whereas others showed more subtle motor deficits only during testing. During this acute episode, 70% of the mice died, but the remainder recovered from the disease. Signs of motor deficits were less obvious after 4 weeks. Histological examination of the brain and spinal cord at 7 days post-inoculation revealed scattered infiltrations of inflammatory cells. C3H mice, however, showed few perivascular infiltrates³. After 14–21 days, numerous focal lesions exhibited vacuolation and fragmentation of the myelin sheaths. Naked axons, necrotic cells and macrophages containing myelin debris were evident around the lesions. Mild perivascular infiltration with mononuclear inflammatory cells was also seen in these plaques of demyelination. Such demyelinating lesions were present in almost every section of the spinal cords examined and were situated in the central, lateral or posterior columns. Immunocytochemistry revealed a significant decrease in MBP in these lesions, the remaining stain being associated with myelin debris. After 4 weeks, the demyelinated foci were more prominent and most of the degenerated myelin had been phagocytosed (Fig. 1a). A few partially demyelinated axons with abnormally thin myelin sheath were scattered in the lesion at this time. Ten weeks after infection, remyelination was prominent and most axons in the previously demyelinated areas were surrounded by thinner than normal myelin sheaths (Fig. 1b). Immunostaining showed that MBP had reappeared in these areas and was associated with the newly formed myelin sheath. Invasion of the CNS by Schwann cells to remyelinate bare axons^{20–22} was not detected.

We next examined the cryostat sections of the spinal cord of animals at different stages of the disease after hybridization with the MBP-specific probe and autoradiography (Fig. 2). Since the sections were also stained with cresyl violet, we compared the distribution of the grains (corresponding to the amount of hybridization with the MBP cDNA probe) with the distribution of the lesions. In spinal cord of uninfected mice, more grains were seen in the white matter than in the grey matter, as expected from dot-blot hybridization studies of 1-month-old mice⁵ (Table 1). An unrelated probe (a cDNA of the non-structural phosphoprotein gene of vesicular stomatitis virus²³) showed no significant hybridization to grey or white matter in either normal or infected animals. With the MBP-specific probe, however, a slight increase of labelling of the white matter was detected at 2 weeks (Fig. 2b), and fewer grains were seen in areas of white matter inflammation (Fig. 2a, b). At 3–4 weeks post-inoculation (Fig. 2c–f), the intensity of labelling had increased dramatically in some areas of the white matter. When the distribution of the grains in dark field was compared with the size and location of the lesion as seen after cresyl violet staining (Fig. 2a, c, e, g), it became clear that the foci with inflammation and tissue degeneration were devoid of labelling. However, a striking increase in the number of grains was present at the edge of the lesion as well as in the surrounding, normal-appearing white matter of the entire column (Fig. 2d) and even in the opposite column (Fig. 2f). Animals examined 8 weeks after inoculation had slightly higher levels of labelling in the white matter than had the normals (Table 1), but there were no clearly demarcated unlabelled areas such as those seen during the peak of demyelination (Fig. 2g, h).

The evidence presented here suggests that, during a demyelinating process caused by a virus in rodents^{3,4,8–15}, an increase in transcription of the gene coding for MBP, a major myelin protein, occurs early when demyelination is still progressing. Similarly, in CNS demyelinating lesions caused by cuprizone in rats, MBP can be detected by immunocytochemistry in oligodendrocytes before remyelination²⁴. However, as MBP staining is also seen in macrophages containing myelin debris, it is difficult to distinguish between 'old' MBP and newly synthe-

sized MBP in these lesions. In contrast, *in situ* hybridization with a MBP-specific probe has revealed an approximate 10-fold increase in the number of MBP transcripts in a widespread area surrounding the demyelinating lesions 3–4 weeks after inoculation (Table 1). In normal rodents only basal levels of MBP-specific RNA are expressed at this stage in development^{5,7}. Thus, *in situ* hybridization may be the method of choice for detecting the effects of a demyelinating process on myelin protein gene expression. At 2 months post-inoculation, the remyelinated areas showed only a slight increase in MBP mRNA in a diffuse area including the lesion. This suggests that myelin-forming cells have repopulated the demyelinated area and are completing the synthesis of MBP mRNA at that time. It also implies that, in the adult rodent CNS, a cell of the glial lineage may be capable of dividing and probably of migrating into the lesion to repair myelin^{25,27}. Transplantation studies have recently shown that myelinating cells can migrate significant distances in the CNS²⁸. Earlier electron microscope and autoradiographic studies have shown that the cells associated with remyelination after a viral

Table 1 Quantitation of MBP mRNA levels in spinal cord sections of MHV-A59-infected animals using *in situ* hybridization

Day post-inoculation	0	7	14	20	28	60
No. of mice	4	1	3	4	4	2
Degree of labelling	+	+	++	++++	++++	++

The appearance of MBP mRNA was quantified by counting grains over the white matter using a scaled reticule and rates as follows: +, 2–3 times more grains over white than grey matter; ++, 3–6 times more grains over white than grey matter; +++++, 10 times more grains over white than grey matter.

disease are newly generated oligodendroglia¹¹. Moreover, recent studies on newborn rat optic nerve have demonstrated the existence of a progenitor cell which, after a number of mitoses, can differentiate into an oligodendrocyte²⁹. In the adult CNS, similar progenitor cells have been identified in the optic nerve and in the rat cerebral cortex^{25–27}. Thus, it is now necessary to improve the resolution of our *in situ* hybridization method and combine this approach with the use of cell-specific markers in order to identify precisely the cell type responsible for remyelination in the CNS.

To our surprise, the increase in MBP gene transcription seemed to radiate from the edge of the plaque, far away into the surrounding normal white matter. This suggests that, in this virus-induced demyelinating disease in mice, a factor may be produced in the lesion which could diffuse into the normal white matter and trigger oligodendrocytes and/or their progenitor cells²⁹ to participate in myelin repair^{11,25–27}. One possibility is that such a factor is secreted by inflammatory cells in the lesion, since it has been shown that spleen cells in mice^{30,31} and T lymphocytes in humans³¹ produce factors which promote proliferation and maturation of astrocytes and oligodendrocytes *in vitro*. Moreover, interleukin-2 was recently shown to enhance MBP expression in cultured oligodendrocytes.

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Tumour necrosis factor α stimulates resorption and inhibits synthesis of proteoglycan in cartilage

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During inflammatory reactions, activated leukocytes are thought to produce a variety of small proteins (cytokines) that influence the behaviour of other cells (including other leukocytes). Of these substances, which include the interleukins, interferons and tumour necrosis factors (TNFs), interleukin-1 (IL-1) has been considered potentially a most important inflammatory mediator because of its wide range of effects (reviewed in refs 1, 2). *In vivo* it is pyrogenic and promotes the acute phase response; *in vitro* it activates lymphocytes³ and stimulates resorption of cartilage⁴ and bone^{5,6}. Cartilage resorption is a major feature of inflammatory diseases such as rheumatoid arthritis, and IL-1 is the only cytokine hitherto known to promote it. TNFs are characterized by their effects on tumours and cytotoxicity to transformed cells⁷⁻⁹, but share some actions with IL-1. I report here that recombinant human TNF α stimulates resorption and inhibits synthesis of proteoglycan in explants of cartilage. Its action is similar to and additive with IL-1, and it is a second macrophage-derived cytokine whose production in rheumatoid arthritis, or inflammation generally, could contribute to tissue destruction.

Two human TNFs have been isolated: TNF α (refs 8, 9) is a product of activated mononuclear phagocytes, TNF β (ref. 7) of activated lymphocytes. The proteins show about 50% homology in nucleotide sequence and compete for a common class of receptors on the cervical carcinoma line ME-180 (ref. 10). TNF α is probably identical with cachectin¹¹, a factor that suppresses production of lipoprotein lipase in cultured adipocytes, and may play a part in the development of cachexia during infection¹². IL-1 shows some biological similarity to TNF: it is cytotoxic to certain transformed cells¹³ and it suppresses production of lipoprotein lipase in adipocytes¹⁴. Furthermore, cachectin has recently been shown to stimulate production of prostaglandins and latent collagenase by human synovial and dermal fibroblasts in a manner similar to IL-1¹⁵. In view of these findings, I have investigated the effect of TNF α on both the resorption and synthesis of proteoglycan by explants of cartilage.

Chondroitin-sulphate-rich proteoglycan is an essential component of the matrix of cartilage since it enables the tissue to resist compression during load-bearing. Loss of proteoglycan, such as occurs in rheumatoid arthritis, osteoarthritis and other joint diseases, results in severe impairment of the function of cartilage. IL-1 is the only purified cytokine known to cause cartilage to degrade its proteoglycan^{4,16}, and to inhibit resynthesis¹⁷.

Figure 1a shows the amount of proteoglycan (measured as percentage of total chondroitin sulphate) released from porcine articular cartilage during 6 days of culture in the presence of human recombinant TNF α or pure porcine IL-1. The TNF α caused up to 75% of the proteoglycan to be released, although it was less potent than the IL-1, which was significantly active at a 20-fold lower dose (0.5 pM). Figure 1b shows a similar experiment carried out on cartilage from bovine nasal septum which was cultured for a shorter period (48 h): again, the IL-1 was more potent. The time dependence of the release of proteoglycan from bovine cartilage caused by sub-maximal concentrations of the two agents revealed that their effects were additive. Figure 1c shows that 50 pM IL-1 or 290 pM TNF α caused a similar rate of release, and that this was approximately doubled when the agents were combined. Maximal stimulation of cartilage by IL-1 caused more rapid release of proteoglycan than did TNF α (Fig. 1d): results for two concentrations of each cytokine demonstrate that responses were maximal. Supra-maximal doses of the two agents in combination caused a rate of release that was considerably faster than that due to TNF α alone, but was not significantly greater than that seen with IL-1 alone. The failure of TNF α to augment the maximal response to IL-1 may be because the limit of the chondrocytes' ability to degrade their matrix *in vitro* was being approached.

The enzymatic mechanism by which the proteoglycan is degraded in cartilage is not understood. Normally, cartilage proteoglycans aggregate in a specific manner with hyaluronic acid, and it is thought that the large size of these aggregates causes them to be trapped in the matrix. Cartilage stimulated by IL-1 releases fragments of proteoglycan which, as judged by gel filtration, are smaller than normal proteoglycan monomers and are unable to aggregate with hyaluronic acid¹⁸. There is no evidence of degradation of their chondroitin sulphate chains. These changes suggest that degradation is by limited proteolysis of the protein core. The fragments of proteoglycan that were released by cartilage stimulated with TNF behaved similarly on gel filtration to those generated by stimulation with IL-1 (Fig. 2). The bulk of the fragments generated by stimulation with either agent emerged from a Sepharose 2B column at a region between the elution positions of intact proteoglycan and proteoglycan digested with papain (which consists largely of single-chain chondroitin sulphate peptides). Addition of hyaluronic acid to the proteoglycan fragments before chromatography caused little or no formation of aggregates. This suggested that the hyaluronate binding region was blocked or had been lost. When the proteoglycan fragments were chromatographed under dissociative conditions (4 M guanidine-HCl in the chromatographic buffer) the position of the main peak was unchanged. These experiments showed that chondrocytes activated by TNF or IL-1 caused a similar limited proteolysis of the proteoglycans.

In order to study the effect of TNF α on the synthesis of proteoglycan, cartilage was stimulated for 48 h, and ³⁵SO₄ was added to the culture medium for the last 6 h. In this procedure the isotope becomes incorporated into newly synthesized sulphated glycosaminoglycan (mainly chondroitin sulphate). At the end of the experiment the medium and cartilage were digested with papain, and glycosaminoglycan was precipitated from the digests with cetylpyridinium chloride. The amount of radioactivity present in the precipitates was a measure of chondroitin sulphate (and, by inference, proteoglycan) synthesis. In experiments made with porcine articular (Fig. 3a) or bovine nasal septal (Fig. 3b) cartilages, TNF α caused a marked sup-

Fig. 1 Stimulation of release of proteoglycan from cartilage by $\text{TNF}\alpha$ or IL-1. *a*, Porcine articular cartilage. The amount of chondroitin sulphate released was expressed as a percentage of the total (the content of the medium and tissue combined)²¹. Results are shown as means of quintuplicate cultures $\pm$ s.e.m. $\bullet$, TNF ; $\circ$, IL-1; $\square$, no addition. *b*, Bovine nasal septal cartilage. Symbols as for *a*. *c*, Time course for bovine cartilage disks cultured as in *b* with no addition ($\square$), 50 pM IL-1 ($\circ$), 290 pM TNF ($\bullet$), 50 pM IL-1 and 290 pM TNF in combination (Δ). Chondroitin sulphate released was measured at the indicated times and results are means $\pm$ s.e.m. of eight individual disk cultures. *d*, Time course for bovine cartilage disks cultured as in *c*, with no addition ($\square$), 30 nM TNF ($\bullet$), 90 nM TNF ($\blacklozenge$), 1.5 nM IL-1 ($\circ$), 4.5 nM IL-1 ($\diamond$) and 90 nM TNF and 4.5 nM IL-1 in combination (Δ).

Methods. *a*, Pieces of articular cartilage were removed from the metacarpal heads of freshly slaughtered young pigs. Pieces (~4 mg wet weight) were maintained for 48 h at 37°C in CO_2 /air 1:19 in culture medium [Dulbecco's modified Eagle's medium (DMEM)] containing 5% normal bovine serum that had been heat-inactivated at 56°C for 30 min. Each was then transferred to a well of a 96-well multititre plate and incubated under the same conditions in 0.2 ml of culture medium, either with no addition, or with human $\text{TNF}\alpha$ or porcine IL-1 of pI 5. The medium was changed at 3 days and the culture was terminated after 6 days. Human $\text{TNF}\alpha$ was a recombinant protein expressed in *Escherichia coli* and purified as described previously^{7,22}. Porcine IL-1 was a natural leukocyte protein purified to homogeneity as described elsewhere¹⁶. The pI 5 form rather than the pI 8 form was used for these experiments. After culture the medium and cartilage were separated. The cartilage was digested completely with papain (see legend to Fig. 3). The chondroitin sulphate content of this digest and culture medium was estimated by use of the metachromatic dye, dimethylmethylene blue (Serva); whale chondroitin sulphate (Sigma) was used as a standard. *b*, Disks (2 $\times$ 1 mm) of cartilage were cut from the bovine nasal septa of freshly killed animals. Disks were precultured for 48 h and then stimulated for 48 h with $\text{TNF}\alpha$ or IL-1. The chondroitin sulphate content of medium samples and tissue digests was measured exactly as described for pig particular cartilage.

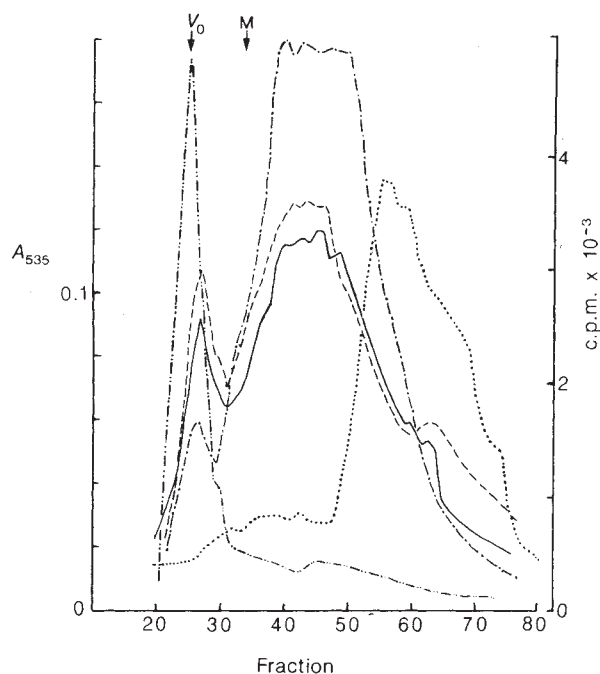
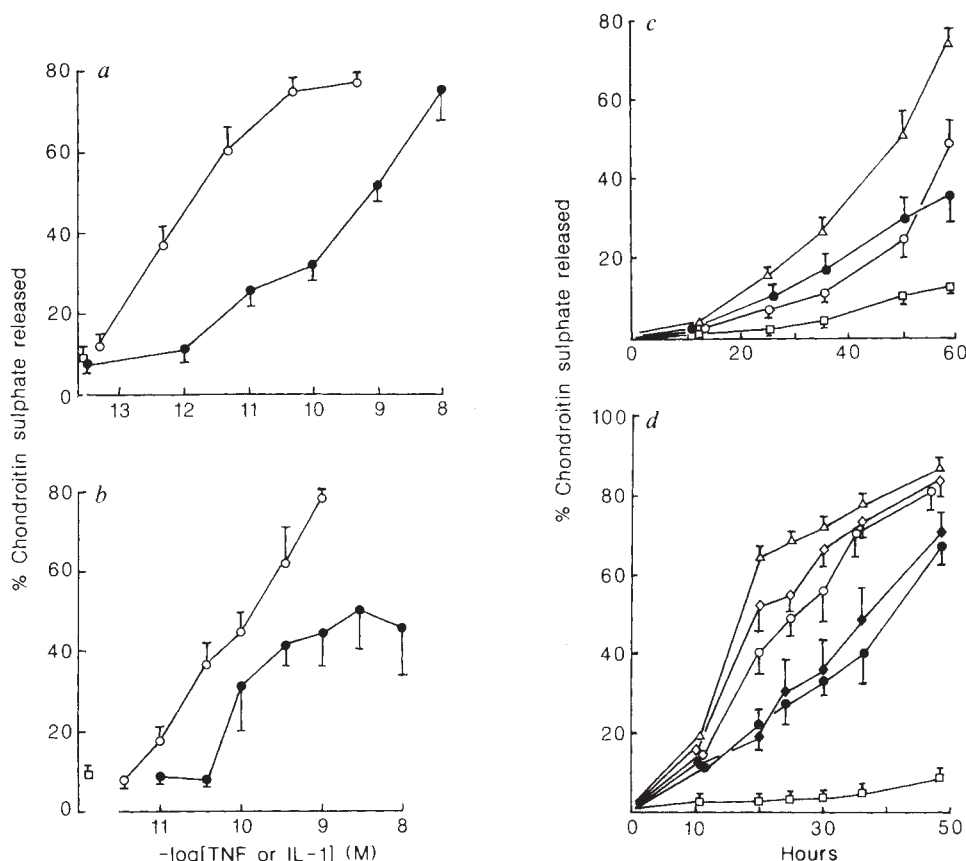


Fig. 2 Gel chromatography of proteoglycans released from stimulated bovine nasal cartilage. Samples of culture supernatants from stimulated bovine nasal cartilage were chromatographed on a column (930 $\times$ 6.5 mm) of Sepharose 2B (Pharmacia) eluted with 0.5 M acetate buffer pH 5.8. Fractions (0.4 ml) were collected. Proteoglycan was detected as chondroitin sulphate by the dye dimethylmethylene blue (see Fig. 1) and is shown as A_{535} . —, Culture supernatant from bovine nasal cartilage cultured as in Fig. 1 in the presence of 5 nM TNF ; ----, the same supernatant to which hyaluronic acid (2%) had been added before chromatography; - - - -, culture supernatant from cartilage stimulated with 0.5 nM porcine IL-1, and to which hyaluronic acid had been added before chromatography;, a papain digest of proteoglycan extracted from fresh cartilage by 4 M guanidine-HCl. ---, proteoglycan (+2% hyaluronic acid) extracted by guanidine-HCl (4 M) from unstimulated cartilage that had been biosynthetically labelled with $^{35}\text{SO}_4$ for 24 h, measured as c.p.m. Arrows are: V_0 , void volume; M, elution position of $^{35}\text{SO}_4$ -labelled proteoglycan without addition of hyaluronic acid.

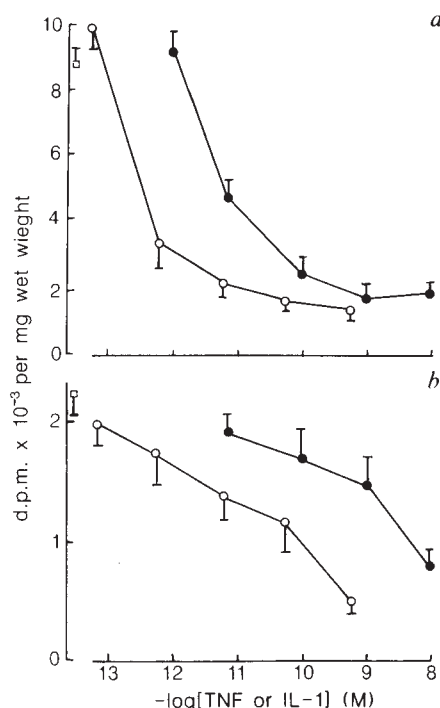


Fig. 3 Effects of TNF α or IL-1 on the incorporation of ³⁵SO₄ into glycosaminoglycans of cartilage. *a*, Porcine articular cartilage; *b*, Bovine nasal septal cartilage. Each assay point is the mean of five separate explants $\pm$ s.e.m. ●, TNF α ; ○, IL-1; □, no addition. **Methods.** *a*, Pieces of pig articular cartilage were dissected, precut and then stimulated either with TNF α or porcine IL-1 for 48 h exactly as described for Fig. 1, except that the culture medium (DMEM) contained 1% normal bovine serum (heat inactivated) during the stimulation period. For the last 6 h the medium was replaced with SO₄-free culture medium (still containing TNF α or IL-1) to which was added 2.5 μ Ci ml⁻¹ of ³⁵SO₄ (25–40 Ci mg⁻¹; Amersham). After culture, the cartilage pieces were separated from the medium, briefly blotted to remove excess medium, and then weighed. Each piece was digested at 65 °C for 2 h in 0.2 ml of 0.05 M sodium phosphate buffer pH 6.5 containing 1 mM EDTA, 2 mM N-acetylcysteine, 28 μ g ml⁻¹ papain (Sigma Type III). Samples of medium (0.2 ml) were also digested with 0.1 ml of the papain solution under the same conditions. Chondroitin sulphate (0.1 ml of 2 mg ml⁻¹) was added to all the samples followed by 0.1 ml of cetylpyridinium chloride (10% w/v). Samples were centrifuged, the precipitates were washed twice in 3% cetylpyridinium chloride, then dissolved in 0.5 ml of formic acid and added to 5 ml of a scintillation mixture (Pico-fluor-30, Packard) and counted in a liquid scintillation counter. The radioactivity of the digests of tissue and medium were added together and the results expressed as d.p.m. per mg wet weight of cartilage. *b*, As for *a* except that disks of bovine nasal septal cartilage were used (see Fig. 1), and the culture medium contained 5% normal bovine serum throughout the experiment.

pression of proteoglycan production as judged the incorporation of ³⁵SO₄ into glycosaminoglycan, but was less potent than IL-1. The porcine IL-1 inhibited incorporation in porcine articular cartilage in the range 0.1–10 pM; TNF α was 20-fold less active. A similar differential was observed on the bovine cartilage. The lower potency of the human TNF α compared with porcine IL-1 may be due to species differences.

Taken together, the experiments demonstrate that TNF α has a similar action to IL-1 on chondrocytes. It causes them to degrade proteoglycan by limited proteolysis and inhibits their synthesis of new proteoglycan. Exposure of cartilage to either of these leukocyte products during inflammation could lead to loss of proteoglycan and impairment of function; furthermore, their effects may be additive.

Pigs¹⁶, like humans¹⁹, have two different IL-1 proteins: for these experiments the pI 5 IL-1 was used rather than the pI 8

form. The IL-1s are equipotent on cartilage¹⁶ and compete for the same receptors on porcine synovial fibroblasts: TNF α at 400 times excess over IL-1) did not compete for these receptors (T. A. Bird and J. S., in preparation). The augmentation of the effect of maximal doses of TNF α by IL-1 reported here is consistent with there being different receptors on chondrocytes for the two cytokines. Since they are apparently not homologous^{8,16}, IL-1 and TNF α would be expected to combine with different receptors. These considerations suggest that chondrocytes (and probably other connective tissue cells) could have two distinct types of receptor (one for IL-1 and one for TNF) whose interaction with ligand promotes resorption of matrix polymers while inhibiting their synthesis. Such a possibility could have important implications for the pharmacological control of inflammatory tissue destruction.

Following submission of this manuscript, Bertolini *et al.*²⁰ have reported that human TNFs, like IL-1, stimulated Ca²⁺ release from fetal rat bones.

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Post-translational insertion of a fragment of the glucose transporter into microsomes requires phosphoanhydride bond cleavage

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Most eukaryotic secretory and membrane proteins insert co-translationally into the membrane of the rough endoplasmic reticulum (RER), and are targeted there by one or more NH₂-terminal or internal signal sequences (for reviews see refs 1, 2). However, little is known about the actual translocation and membrane integration processes. In particular, any energy requirements for targeting and integration have remained obscure because of the inability to uncouple the processes from concomitant protein synthesis. We

recently showed that the human glucose transporter (GT), an integral membrane glycoprotein³, can insert post-translationally into dog pancreatic microsomes with low but demonstrable efficiency *in vitro*, and that a fragment corresponding to the NH₂-terminal 340 amino acids and 8 of the 12 membrane-spanning α -helices of GT (GT-N) can insert with significantly greater efficiency⁴. We report here that post-translational insertion of GT-N into pancreatic microsomes requires energy in the form of a phosphodiester bond, and suggest that co-translational insertion of proteins into the RER may also require energy independent of that used for polypeptide synthesis.

From experiments using *in vitro* translation and membrane translocation⁵⁻⁸, Walter and Blobel^{1,8} proposed the following sequence of events for the targeting of proteins for translocation across the RER membrane. Initiation of synthesis of a secretory polypeptide starts on a messenger RNA-ribosome complex free in the cytosol. The emergence of an N-terminal signal sequence from the large ribosomal subunit triggers the binding of cytosolic signal recognition particle (SRP) and arrest of polypeptide chain elongation. The complex then binds to the surface of the RER via a transient interaction between SRP and the membrane-bound SRP receptor⁹⁻¹¹ (docking protein¹²). Integral membrane proteins¹³⁻¹⁷ which may possess one or more^{4,13} N-terminal or internal signal sequences also utilize this targeting process, although specific arrest of polypeptide chain elongation by SRP does not always occur during translation *in vitro*^{4,14}. Subsequent events in the translocation/integration process remain obscure, particularly whether the nascent chain traverses the membrane through an aqueous pore^{5,18} or directly through the fatty acyl core of the lipid bilayer¹⁹.

To investigate the energetics of membrane protein insertion, we used a recently developed *in vitro* assay in which efficient insertion into RER membranes of a fragment of the human glucose transporter, corresponding to the NH₂-terminal 340 amino acids (GT-N), is uncoupled from protein synthesis⁴.

RNA was transcribed from a SP65 plasmid construction encoding GT-N and used as a template for the synthesis of GT-N in a reticulocyte cell-free translation system. GT-N can insert post-translationally into dog pancreatic microsomes with a conformation resembling that of the corresponding region of the native membrane-bound protein⁴. Figure 1a shows that 50% of GT-N inserted into microsomes is added post-translationally, as monitored by the appearance of the 36,000 (36K) relative molecular mass (M_r) glycosylated form of the polypeptide (GT-N₁) (Fig. 1a, lane 2). Additionally, trypsin digestion of pancreatic microsomes generates the appropriate fragment containing the first six membrane-embedded helices of GT^{3,4} (Fig. 1b, lane 8). The addition of glucose (Fig. 1a, lane 3), hexokinase (lane 4) or glucose 6-phosphate (lane 6) alone had a negligible effect on post-translational insertion; in contrast, depletion of ATP by the addition of both glucose and hexokinase effectively abolished insertion (Fig. 1a, lane 5, Fig. 1b, lanes 3, 6). Because yeast hexokinase can use other purine nucleoside triphosphates as cofactors, although with less efficiency than when using ATP²⁰, we cannot conclude that the observed effect is due solely or directly to the depletion of ATP.

Figure 1b shows that post-translational insertion of GT-N in ATP-depleted extracts could be reconstituted by the addition of excess creatine phosphate. This is demonstrated by the appearance in pelleted microsomes of glycosylated GT-N after the microsomes have been washed in a neutral buffer (Fig. 1b, compare lane 4 with lanes 2 and 3). When the microsomes are washed in buffer at alkaline pH, peripherally associated membrane proteins as well as the vesicle contents are extracted, so that only integral membrane proteins sediment with the microsomes²¹⁻²³. By this criterion, insertion of GT-N into the lipid bilayer was restored after addition of excess creatine phosphate (Fig. 1b, compare lane 7 with lanes 5 and 6). This is also demonstrated by the generation of the glycosylated 22K NH₂-terminal fragment of GT⁴ after the addition of excess creatine

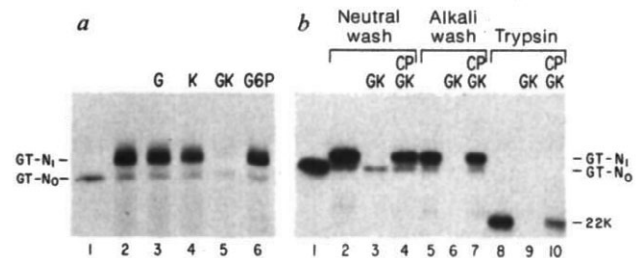


Fig. 1 Membrane insertion of GT-N is inhibited by depletion of nucleoside triphosphates. Synthesis of GT-N mRNA using SP6 RNA polymerase and synthesis of ³⁵S-methionine-labelled GT-N in a reticulocyte cell-free system have been described previously⁴. Translation was allowed to proceed at 30 °C for 90 min (45 min after net protein synthesis was complete). **a**, After translation 1 mM cycloheximide was added, sufficient to inhibit incorporation of ³⁵S-methionine into GT-N protein by >99% (data not shown). The incubation mixture was divided into five 25- μ l aliquots and then incubated at 30 °C for 10 min without any additions (lane 2), with 5 mM glucose (lane 3), 1.4 U of yeast hexokinase (lane 4), 5 mM glucose and 1.4 U of hexokinase (lane 5), or with 5 mM glucose 6-phosphate (lane 6). Dog pancreatic rough microsomes (3 equiv.²⁹) were then added and incubation was continued for 20 min. The microsomes were sedimented by centrifugation for 20 min in an Eppendorf microfuge, resuspended in 50 μ l of 0.25 M STBS (0.25 M sucrose, 10 mM Tris-Cl pH 7.5, 150 mM NaCl), and re-sedimented. The membrane pellets were dissolved directly in sample buffer and analysed on a 12% polyacrylamide gel using the systems of Laemmli³⁰. Aliquots (5 μ l) of translation reaction to which microsomes were not added were analysed in lane 1; this is the 100% value used here and in subsequent figures to calculate per cent insertion. The gel was washed for 30 min in 10% trichloroacetic acid, 30% isopropanol, treated with Enlightening autoradiography enhancer (Dupont-NEN), dried and exposed to Kodak XAR-5 film for 1 h. **b**, After translation the reaction was adjusted to 1 mM cycloheximide and 50 μ g ml⁻¹ of RNase A and then divided into 20- μ l aliquots. Samples were incubated as described above with no additions (lanes 1, 2, 5, 8) or after addition of 5 mM glucose and 1.4 U of hexokinase (lanes 3, 4, 6, 7, 9, 10). Creatine phosphate (20 mM) was then added to some samples (lanes 4, 7, 10) followed by the addition of microsomes (3 equiv.²⁹ per 20- μ l reaction; lanes 2-10). After 20 min incubation, the microsomes were sedimented as described above and washed in 0.25 M STBS (lanes 2-4 and 8-10) or 0.25 M STBS adjusted to pH 11.5 with NaOH (lanes 5-7), then analysed as described above. Aliquots (5 μ l) of translation reaction to which microsomes were not added were analysed in lane 1. Abbreviations used are: G, 5 mM glucose; K, 1.4 U hexokinase; GK, glucose and hexokinase; CP, 20 mM creatine phosphate; G6P, 5 mM glucose 6-phosphate; GT-N₀, unglycosylated GT-N; GT-N₁, glycosylated GT-N.

phosphate followed by trypsin digestion of the microsomes (Fig. 1b, compare lane 10 with lanes 8 and 9).

After depletion of ATP under the conditions used in these experiments, variable amounts of unglycosylated GT-N sedimented with washed microsomes (Fig. 1a, lane 5; Fig. 1b, lane 3). Only a small fraction of this is due to nonspecific pelleting of GT-N in the absence of microsomes (data not shown). Thus, we do not know whether targeting of GT-N to microsomes is energy dependent.

Figure 2 shows that, after translation, removal of low- M_r components from the lysate by gel filtration inhibits post-translational insertion, as monitored by the appearance of glycosylated GT-N associated with the microsomes (Fig. 2, compare lanes 2 and 3). Addition of 1 mM Mg-ATP (Fig. 2, lane 4) or 10 mM Mg-ATP (Fig. 2, lane 5) to the lysate after gel filtration restored the appearance of glycosylated GT-N. The non-hydrolysable β , γ -methylene ATP analogue did not promote insertion of GT-N, and inhibited the residual insertion observed after gel filtration of the lysate (Fig. 2, compare lane 6 with lanes 2 and 3).

In this assay, ATP and GTP are equally effective in promoting insertion; UTP and CTP are less effective (data not shown).

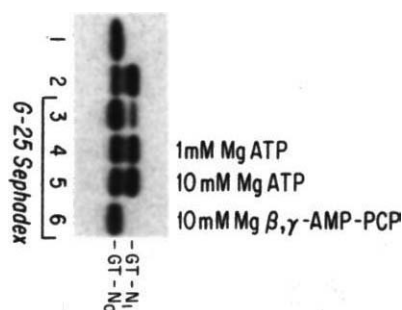


Fig. 2 ATP restores membrane insertion of GT-N in gel-filtered lysates. After synthesis of GT-N in a reticulocyte lysate, as described in Fig. 1 legend, the lysate (100 μ l) was adjusted to 1 mM cycloheximide and centrifuged through two successive 0.9 ml Sephadex G-25 columns equilibrated in column buffer (50 mM HEPES pH 7.5, 5% glycerol, 0.1 M KAc, 5 mM MgAc, 2 mM dithiothreitol, 1% bovine serum albumin, 1 mM TPCK and TLCK, 1 mM cycloheximide). The gel-filtered lysate was then treated with 50 μ g ml⁻¹ RNase A for 10 min at 30 °C. Microsomes were then added (3 equiv.²⁹) to 25- μ l aliquots without any additions (lane 1) or with 1 mM MgAc-ATP (lane 2), 10 mM MgAc-ATP (lane 3) or 10 mM MgAc-AMP-PCP (lane 6) and incubated for an additional 30 min. The microsomes were sedimented by centrifugation in an Eppendorf microfuge for 20 min, resuspended and washed in 0.25 mM sucrose, 50 mM Tris-Cl pH 7.5, 25 mM KAc, 5 mM MgAc, and analysed by polyacrylamide gel electrophoresis as described in Fig. 1 legend. Aliquots (5 μ l) of lysate to which microsomes were not added were analysed in lane 1. A microsomal fraction added to 25 μ l of unfiltered lysate was analysed in lane 2.

Because enzymes that catalyse the transfer of phosphate from one nucleotide to another (for example, nucleoside diphosphate kinase) may well be present in these crude extracts, and the removal of low- M_r components may not be quantitative, we do not know which nucleotide (or other high-energy phosphate compound) is the proximal source of energy.

Under the washing conditions used in this experiment (25 mM potassium acetate, 5 mM magnesium acetate), a considerable fraction of unglycosylated GT-N sedimented with the microsomes. Only a small fraction of the non-glycosylated GT-N was inserted, since most could be removed by alkali extraction (data not shown). Whether the alkali-labile fraction represents a genuine intermediate in membrane insertion, or simply an adventitious association of the unintegrated protein with the microsomes, is not known.

The GT-N mRNA used in these experiments lacks a termination codon, so that proper polypeptide chain termination and release of the completed polypeptide should not occur. Surprisingly, Fig. 3 shows that about 75% of GT-N synthesized was recovered in a post-ribosomal supernatant (compare lanes 2 and 3) and presumably represents completed and released polypeptides. The remaining 25% of GT-N sedimented with ribosomes and presumably is a completed peptidyl-transfer RNA-ribosome complex. Supporting this latter point is the observation that the yield of GT-N in the ribosomal fraction was reduced by at least 50% if the lysate was incubated with puromycin before sedimenting the ribosomes (data not shown). In this experiment about 25% of GT-N in the unfractionated lysate inserted post-translationally into the microsomes (Fig. 3, lane 4). The GT-N in the post-ribosomal supernatant did not insert (Fig. 3, lane 5). As monitored by the appearance of glycosylated GT-N, very little GT-N in the resuspended ribosomal pellet inserted in the absence of added ATP (Fig. 3, lane 6). Insertion was increased by including ATP in the buffer (Fig. 3, lane 7). Adding a ribosome fraction obtained from reticulocyte lysate not programmed with mRNA to the GT-N in the post-ribosomal supernatant did not reconstitute insertion, indicating that the lack of insertion of released GT-N was not due to the lack of

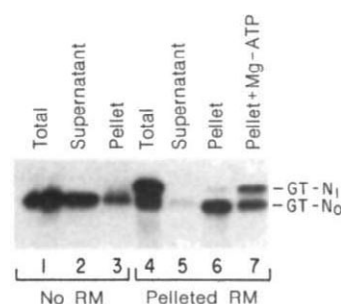


Fig. 3 Only GT-N associated with ribosomes acts as a substrate for membrane insertion. GT-N was synthesized in a reticulocyte lysate as described in Fig. 1 legend. The lysate was then adjusted to 1 mM cycloheximide and fractionated into a ribosomal pellet and a post-ribosomal supernatant by centrifugation for 30 min at 300,000 g in a Beckman TL-100.2 rotor. The ribosomal pellet was resuspended in a volume of column buffer (see Fig. 2 legend) equal to that of the original lysate. Rough microsomes (RM, 3 equiv.²⁹) were then added to aliquots of the unfractionated lysate, post-ribosomal supernatant and resuspended ribosomes. The sedimented microsomal fractions were then washed and analysed by polyacrylamide gel electrophoresis as described in Fig. 2 legend. Lane 1, 5 μ l of unfractionated lysate; lane 2, 5 μ l of post-ribosomal supernatant; lane 3, 5 μ l of resuspended ribosomes; lane 4, microsomes from 25 μ l of unfractionated lysate; lane 5, microsomes from 25 μ l of post-ribosomal supernatant; lane 6, microsomes from 25 μ l of resuspended ribosome fraction; lane 7, microsomes from 25 μ l of resuspended ribosome fraction supplemented with 5 mM MgAc-ATP.

a soluble factor that sedimented with the ribosomes (data not shown). Thus, the behaviour of GT-N is unlike that of the intact GT, which can insert (with a lower efficiency) into microsomes as a completed polypeptide⁴.

One possible explanation for these observations is that glycosylation of GT-N does not occur in the absence of ATP, and that glycosylation is necessary for insertion. Several considerations argue against this interpretation. Insertion of other proteins into membranes both *in vivo* and *in vitro* does not require Asn-glycosylation (see, for example, ref. 24). In our system, glycosylation is unaffected by concentrations of tunicamycin in vast excess of that required to inhibit formation of GlcNAc-P-P-dolichol *in vivo* and *in vitro* (our unpublished observations), indicating that *de novo* synthesis of the dolichol-core oligosaccharide is not required. Furthermore, significant glycosylation of GT-N is observed in the absence of nucleoside sugars (which should not be present at significant levels after gel filtration of lysate or in the resuspended ribosomal fraction: see Figs 2, 3), indicating that elongation of GlcNAc-P-P-dolichol is not required in our system. Thus, sufficient preformed core oligosaccharide-dolichol donor must be present in our microsomal preparation, and transfer of the core oligosaccharide from the dolichol donor to asparagine residues does not require a nucleotide cofactor²⁵.

Our observations suggest that targeting and/or insertion of GT-N into pancreatic microsomes is dependent on energy in the form of a nucleotide triphosphate cofactor. Whether this dependence involves known factors in the targeting process, or an as yet unknown factor or factors involved in translocation, remains to be determined. Since no ionophore tested (monensin, gramicidin, A23187) blocks insertion (not shown), we feel that hydrolysis of a phosphoanhydride bond is not used to generate an electrochemical potential across the ER membrane which is in turn used to power translocation. In this respect, post-translational insertion into RER membranes is different from energy-dependent translocation across the bacterial²⁶ or mitochondrial²⁷ inner membrane and similar to polypeptide import into chloroplasts²⁸. We speculate that co-translational insertion of

membrane and secretory proteins into or across the RER also requires energy in addition to that consumed in protein synthesis. This could involve an ATP- or GTP-driven translocase that is part of a transmembrane channel^{5,11} through which the nascent polypeptide moves.

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Role of ion flux in the control of *c-fos* expression

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There has been much interest in the biochemical and biophysical processes that couple extracellular signals to alterations in gene expression. While many early events associated with the treatment of cells with growth factors have been described (for example, ion flux and protein phosphorylation^{1,2}), it has proved difficult to establish biochemical links to gene expression. Recently, the study of such genomic control signals has been facilitated by the demonstration that the *c-fos* proto-oncogene is rapidly and transiently induced by treatment of several cell types with polypeptide growth factors and other growth modulating substances³⁻⁸. In one particular system it has been shown that nerve growth factor (NGF) causes a transient induction of *c-fos* in the pheochromocytoma cell line PC12, within 15 min⁹⁻¹¹. Furthermore, the magnitude of this induction can be modulated with pharmacological agents such as peripheral-type benzodiazepines (BZDs)⁹. Thus, the study of *c-fos* expression in PC12 cells could yield valuable clues to the coupling mechanisms linking cell surface activation to genomic events. Here we demonstrate that *c-fos* is induced in PC12 cells either by receptor-ligand interaction or by agents or conditions that effect voltage-dependent calcium channels.

In the following experiments the synthesis of the *c-fos* protein has been monitored by immunoprecipitation after metabolic labelling with ³⁵S-methionine. For quantitative analysis, immunoprecipitates from equivalent amounts of cell lysate, as

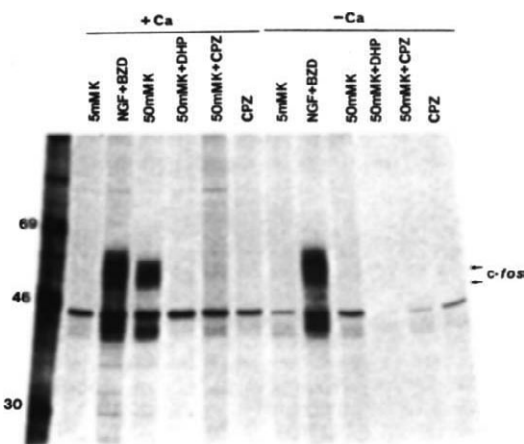


Fig. 1 Induction of *c-fos* protein by 50 mM potassium is calcium- and calmodulin-dependent. PC12 cells were incubated in basal medium with the following additions: 5 mM potassium chloride (5 mM K), 200 ng ml⁻¹ NGF plus 100 μ M benzodiazepine (7-3351) (NGF+BZD), 50 mM potassium chloride (50 mM K), 50 mM potassium chloride plus 15 μ M dihydropyridine (nisoldipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate) (50 mM K+DHP), 50 mM potassium chloride plus 30 μ M chlorpromazine (50 mM K+CPZ) or 30 μ M chlorpromazine (CPZ), in the presence (+Ca) or absence (-Ca) of 1.1 mM calcium chloride. The drugs chlorpromazine and nisoldipine were added to cultures 5 min before the other reagents. In the experiment shown, nisoldipine was used at a saturating concentration (15 μ M), however, 50 nM nisoldipine also attenuated potassium-induced *c-fos* expression (data not shown). Incubation was continued for 30 min at 37 °C then 300 μ Ci ml⁻¹ ³⁵S-methionine ($\approx$ 800 μ Ci mmol⁻¹; Amersham) was added for a further 15 min. Extracts were prepared and immunoprecipitated with *fos*-specific antibodies. The immunoprecipitation products from 10⁷ c.p.m. of TCA-insoluble proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Arrows indicate the position of the *c-fos* protein. The numbers on the left indicate the relative molecular masses (*M*_s) of the ¹⁴C-methylated marker proteins (Amersham). **Methods.** PC12 cells³³ were cultured in RPMI medium (Gibco) containing 10% horse serum, 5% calf serum and 4 mM L-glutamine. For experimentation, cells were seeded onto 35-mm Petri dishes (Falcon) and allowed to attach and proliferate for 48 h. Immediately before experimentation this medium was removed and replaced with 1 ml of incubation buffer (basal medium) or 1 ml of Dulbecco-Vogt modified Eagle's medium (DMEM) lacking methionine. Basal medium comprised 20 mM HEPES, 146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂ and 0.2% D-glucose. Preparation of cell lysates, immunoprecipitation, SDS-PAGE and fluorography were as described previously^{6,34,35}. The *fos*-specific antibodies used were affinity-purified against a peptide predicted from the *c-fos* nucleotide sequence³⁴.

measured by trichloroacetic acid (TCA)-insoluble radioactivity, were compared. Care was taken to ensure that the various treatments did not result in more than a 50% reduction of ³⁵S-methionine incorporation as some inhibitors of protein synthesis can cause an accumulation of *c-fos* messenger RNA^{3,4,6}. Dose-response analyses were performed for all the agents used so as to obtain the maximal specific effect of each.

We demonstrated previously that NGF induces *c-fos* in PC12 cells and that this effect is enhanced in the presence of peripherally active BZDs⁹. Chronic depolarization of PC12 cells due to an increased extracellular potassium concentration has also been shown to influence neurite growth in a calcium-dependent manner^{12,13} and to induce *c-fos* expression^{10,11} (Fig. 1). Thus, we asked whether NGF and elevated K⁺ induce *c-fos* via the same mechanism. Figure 1 shows that the superinduction of *c-fos* by NGF plus benzodiazepine is independent of extracellular calcium, as is NGF induction of *c-fos* (Fig. 2b).

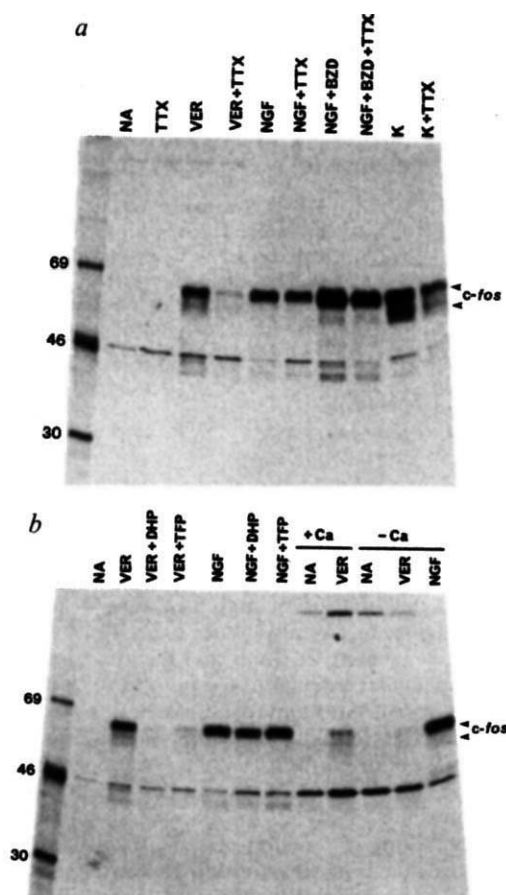


Fig. 2 Veratridine-mediated depolarization induces synthesis of *c-fos* protein. *a*, PC12 cells were incubated in methionine-free DMEM with the following additions: no addition (NA), 3 μ M tetrodotoxin (TTX), 200 μ M veratridine (VER), 200 μ M veratridine plus 3 μ M TTX, 200 ng ml⁻¹ nerve growth factor (NGF), 200 ng ml⁻¹ NGF plus 3 μ M TTX, 200 ng ml⁻¹ NGF plus 100 μ M benzodiazepine (7-3351) (NGF + BZD), 200 ng ml⁻¹ NGF plus 100 μ M benzodiazepine (7-3351) plus 3 μ M TTX (NGF + BZD + TTX), 50 mM potassium (K), or 50 mM potassium plus 3 μ M TTX (K + TTX) for 30 min before labelling with ³⁵S-methionine. *b*, PC12 cells were incubated in methionine-free DMEM containing: no additions (NA), 200 μ M veratridine (VER), 200 μ M veratridine plus 15 μ M dihydropyridine (nisoldipine) (VER + DHP), 200 μ M veratridine plus 15 μ M trifluoperazine (VER + TFP), 200 ng ml⁻¹ NGF, 200 ng ml⁻¹ NGF + 15 μ M dihydropyridine (NGF + DHP), or 200 ng ml⁻¹ NGF plus 15 μ M trifluoperazine (NGF + TFP); in basal medium with: no additions (NA), or 200 μ M veratridine (VER) in the presence of 1.1 mM calcium chloride (+Ca); and in basal medium with no additions (NA), 200 μ M veratridine (VER) or 200 ng ml⁻¹ NGF in the absence of calcium (-Ca). ³⁵S-methionine labelling, preparation of cell lysates, immunoprecipitation and SDS-PAGE were as described in Fig. 1 legend. The numbers on the left indicate the *M_r*s of marker proteins. Arrows indicate the position of the *c-fos* protein. Tetrodotoxin, nisoldipine and trifluoperazine were added 5 min before incubation with the other agents.

In contrast, elevated potassium induces *c-fos* only when calcium is present in the extracellular environment (Fig. 1); this suggests that depolarizing concentrations of potassium induce *c-fos* by provoking an influx of calcium ions, presumably via voltage-dependent calcium channels. This interpretation is supported by the finding that the 1,4-dihydropyridine calcium channel antagonist nisoldipine (DHP)¹⁴ completely blocks potassium-induced *c-fos* expression (Fig. 1), consistent with the known ability of nisoldipine to block potassium-stimulated calcium uptake into PC12 cells¹⁵. In contrast, the induction of *c-fos*

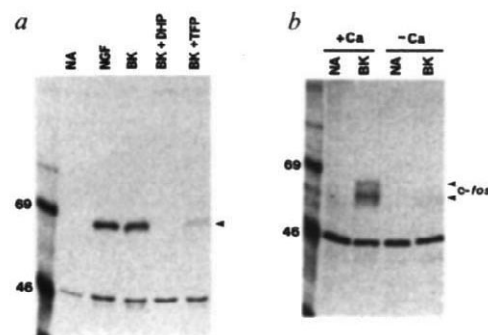


Fig. 3 Stimulation of *c-fos* expression by the calcium channel agonist BAY K 8644. *a*, PC12 cells were incubated in methionine-free DMEM containing: no additions (NA), 200 ng ml⁻¹ NGF, 300 nM BAY K 8644 (BK), 2.5 μ M BAY K 8644 plus 15 μ M dihydropyridine (nisoldipine) (BK + DHP) or 2.5 μ M BAY K 8644 plus 15 μ M trifluoperazine (BK + TFP). *b*, PC12 cells were incubated in basal medium with either no additions (NA) or 2.5 μ M BAY K 8644 (BK) in the presence (+Ca) or absence (-Ca) of 1.1 mM calcium chloride. The numbers on the left indicate the *M_r*s of marker proteins. Arrows indicate the position of the *c-fos* protein. Nisoldipine and trifluoperazine were added (where indicated) 5 min before incubation with the other agents. BAY K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was synthesized by the research division of Hoffmann-La Roche Inc.

protein (NGF + DHP, Fig. 2*b*) and mRNA (data not shown) by NGF is insensitive to dihydropyridine treatment.

The depolarization-induced calcium influx (via the voltage-dependent calcium channels) appears to elicit *c-fos* expression by an interaction with calmodulin since chlorpromazine (CPZ), a calmodulin antagonist¹⁶, blocks the action of elevated potassium (see Fig. 1, 50 mM K + CPZ). This notion was further corroborated using another calmodulin inhibitor, trifluoperazine (TFP)¹⁶, which also blocks K⁺-induced *c-fos* expression (data not shown). These agents did not reduce the level of *c-fos* after treatment with either NGF or 12-*O*-decanoylphorbolmyristate acetate (PMA) (data not shown). As PMA presumably acts via protein kinase C¹⁷, the lack of effect of either CPZ or TFP on this response suggests that the drugs are targeting calmodulin and not protein kinase C, their alternative site of action¹⁸. The more specific calmodulin inhibitor W7 (*N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide¹⁹) clearly attenuated veratridine-induced *c-fos* expression but did not affect NGF induction of *c-fos* (data not shown). Thus, the available evidence points to calmodulin having a role as a mediator in the coupling mechanism of one group of inducers. These observations emphasize further the mechanistic dichotomy between induction of *c-fos* by NGF and by elevated potassium.

An alternative method of depolarizing PC12 cells is to use the alkaloid veratridine which holds voltage-dependent sodium channels in their open state^{20,21}. Veratridine, like elevated potassium, provokes an induction of *c-fos* in a dose-dependent manner (Fig. 2*a* (VER), and data not shown). This action of veratridine is mediated by the well-characterized adult form of the voltage-dependent sodium channel as it is antagonized by tetrodotoxin (TTX)²² (see Fig. 2*a*). It has been reported that PC12 cells do not exhibit voltage-sensitive sodium currents in the absence of NGF²³. Thus, our data may have two interpretations. First, this may reflect a fundamental difference between the PC12 cells in various laboratories. Second, PC12 cells may contain a class of TTX-sensitive sodium channels that can be activated only by veratridine. We noted that tetrodotoxin blocks veratridine-induced *c-fos* expression and is much less effective at reducing the level of *c-fos* observed following treatment with NGF or other agents, thus precluding a nonspecific inhibition

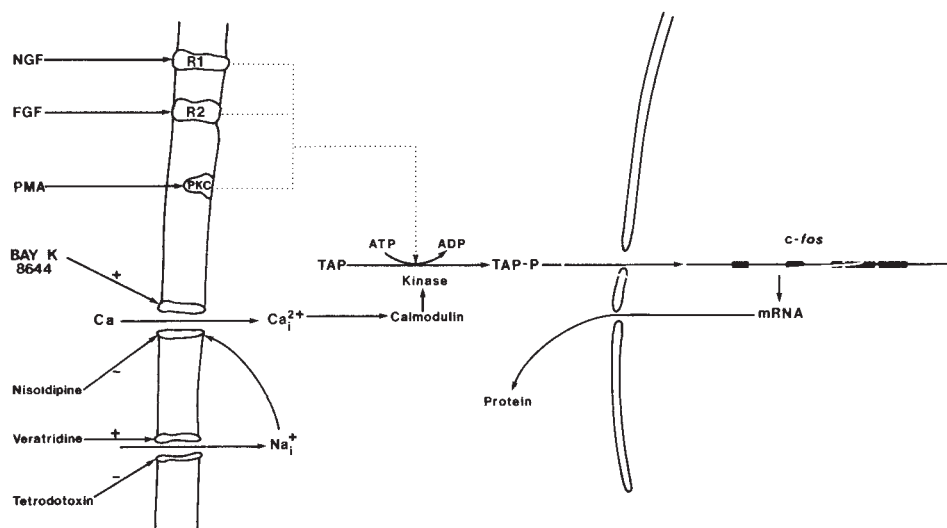


Fig. 4 Schematic representation of the molecular events involved in *c-fos* induction. The figure includes a summary of the data presented in Figs 2 and 3. The action of ion-channel agonists (+) and antagonists (-) is indicated.

(Fig. 2a). Veratridine stimulation of *c-fos* expression, like that of elevated potassium, is also calcium-dependent and is blocked by both nisoldipine and antagonists of calmodulin (Fig. 2b). Thus, depolarization of PC12 cells, either by abolishing the ion gradient for potassium or by specifically holding voltage-dependent sodium channels in their open state, results in *c-fos* induction. The second-messenger coupling mechanism is the same in both instances and seems to involve activation of voltage-dependent calcium channels with a subsequent interaction of calcium with the calmodulin system.

The next question to be addressed is whether the opening of calcium channels alone can lead to induction of *c-fos*. This proposition was tested using the calcium channel agonist BAY K 8644 (ref. 24), which stimulates calcium uptake in PC12 cells²⁵ and is a potent inducer of *c-fos* expression in these cells; at a concentration of 300 nM, it elicits the same response as 200 ng ml⁻¹ of NGF (see Fig. 3a). Stimulation of *c-fos* expression by BAY K 8644 occurs only in the presence of extracellular calcium (Fig. 3b) and is blocked by both nisoldipine and the calmodulin inhibitor trifluoperazine (Fig. 3a). Thus, the action of BAY K 8644 mimics that of extracellular potassium and veratridine treatment both qualitatively and quantitatively. It has been demonstrated recently that BAY K 8644 opens only a limited population of voltage-dependent calcium channels (the so-called long-term or L channels) in dorsal root ganglion neurones²⁶; this suggests that a very specific gated calcium signal is responsible for *c-fos* activation.

The data presented here demonstrate a close link between the biophysical processes involved in neuronal excitation and gene transcription. In particular, the results show that agents or conditions that modify the open time of voltage-dependent calcium channels can modulate expression of the *fos* proto-oncogene. Figure 4 depicts a model of the sequence of events that couple these agents to the transcriptional activation of *c-fos*. Depolarization of PC12 cells with elevated potassium or veratridine results in prolonged opening of voltage-dependent calcium channels, based on three lines of evidence. First, both of these agents require extracellular calcium to induce *c-fos*. Second, both veratridine- and potassium-induced *c-fos* expression are blocked by voltage-dependent calcium channel antagonists such as nisoldipine. Third, a known agonist for the voltage-dependent calcium channel, BAY K 8644, induces *c-fos* directly. Thus, while veratridine and potassium influence the movement of several ionic species across the plasma membrane, the opening of the calcium channel is the common denominator in the cascade of events that result in *c-fos* induction. Activation of the voltage-dependent calcium channel then leads to an influx of calcium ions. This supposition is based on the fact that

veratridine, elevated potassium and BAY K 8644 all require extracellular calcium in order to induce *c-fos*. Thus, activation of the voltage-dependent calcium channel must lead to an increase in intracellular calcium to trigger *c-fos* induction.

All three agents activate calmodulin either as a direct effect of increased calcium levels or by some other indirect route. Calmodulin is implicated because both trifluoperazine and chlorpromazine block *c-fos* induction by veratridine and potassium. As induction by BAY K 8644 is also blocked by calmodulin inhibitors, calmodulin must be involved at a step subsequent to calcium channel activation. Following stimulation of calmodulin, we propose that there is a calmodulin- or calmodulin kinase-mediated modification of a transcription activating protein (TAP). This modified factor then acts, directly or indirectly, to stimulate *c-fos* transcription. Although the latter two steps are speculative there is some evidence to suggest that the *c-fos* gene has an enhancer element which can be activated *in trans* by inducible factors (ref. 27 and T.C., unpublished data). The existence of a similar agent for the haemoglobin gene has been demonstrated in nuclei of stimulated erythroid precursor cells²⁸. In addition, calmodulin has recently been implicated in the control of the prolactin gene²⁹.

Previously, prompted by observations of *c-fos* induction by polypeptide mitogens, a number of investigators suggested that *c-fos* plays a part in controlling cell cycle events. Since then it has been demonstrated that *c-fos* induction is not restricted to a particular phase of the cell cycle and is not necessarily associated with mitogenesis^{7,30,31}. Indeed, in some systems *c-fos* expression has been associated with cellular differentiation^{8,9,11,32}. Our own studies on PC12 cells led us to propose a more general role for *c-fos* in coupling early events associated with receptor occupation (such as phosphorylation, methylation and ion flux) to long-term changes in gene expression⁹. We have now confirmed and extended these data to include an independent biochemical pathway for *c-fos* induction that appears to be particularly prevalent in neuronal cells, and which involves a gated calcium signal. The induction of *c-fos* seems to be associated with, and is a genetic harbinger for, a change in state of many cell types. The change in state may be from quiescence to proliferation or from less to more differentiated for example. We suggest that the target genes for *c-fos* are different in the many and varied situations in which induction occurs. We have shown that agents that classically depolarize neurones lead to an induction of *c-fos* by a gated calcium signal. Thus, we propose that *c-fos* and its co-regulated genes are excellent candidate genes for coupling excitation of the neurone to long-term adaptive modifications of transcription.

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Tumour induction by the retinoblastoma mutation is independent of N-myc expression

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Retinoblastoma (RB) tumours form in the eyes of young children when homozygosity for a mutation at the *Rb-1* locus develops in a somatic retinal cell^{1,2}. A similar shift to homozygosity for the RB mutation has been observed in osteogenic sarcoma (OS) tumours that commonly arise as second tumours in children who survive RB³. This observation suggests that the *Rb-1* locus controls the expression of genes with oncogenic potential; a possible target is the oncogene *N-myc*, which is sometimes amplified and over-expressed in the neuroectodermal tumours neuroblastoma⁴⁻⁷ and RB⁸⁻⁹. However, *N-myc* is developmentally regulated in normal murine embryogenesis⁹, and an alternative possibility is that the expression of the gene in tumour cells reflects their embryonic origin and is unrelated to the RB mutation. We have therefore examined *N-myc* expression in various fetal, adult and tumour tissues, and report here that the gene is expressed in fetal but not in adult brain and retina and in near-diploid RB tumour samples at levels similar to those observed in normal fetal retina. Only RB tumours with genomic amplification of the *N-myc* gene exhibited increased levels of expression; and no *N-myc* transcripts were detected in osteogenic sarcomas initiated by mutations at the *Rb-1* locus³. We therefore conclude that the expression of *N-myc* in RB tumours probably reflects the origin of the tumour from an embryonic tissue normally expressing the gene and is not directly associated with the mutation at the RB locus.

The *Rb-1* locus may have a regulatory function like that of the diploid regulatory/suppressor genes postulated by Comings to control expression of other genes¹⁰. In the absence of the normal RB gene product, such target genes may lead to malignancy because they continue to be expressed at inappropriate levels. *N-myc* is a developmentally regulated proto-oncogene which is a candidate for regulation by a tumour suppressor gene like the RB gene. *N-myc* is normally expressed in the early stages of development in multiple differentiation pathways⁹. Differentiation of neuroblastoma¹¹ and teratocarcinoma cells⁹ in culture is accompanied by a decrease in the level of *N-myc*

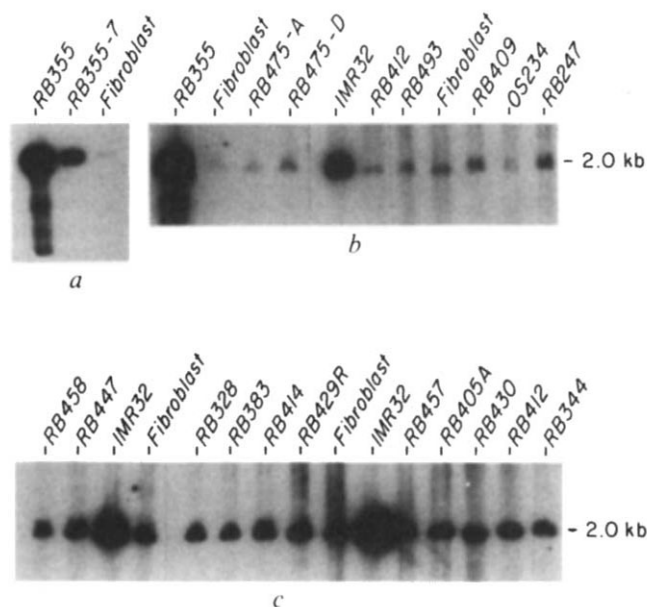


Fig. 1 Southern blot analyses of 17 RB tumours or cell lines and one osteogenic sarcoma cell line (OS234) probed with *N-myc*. DNA was extracted from 5×10^7 – 10^8 cultured cells or tumour cells or tumour material by the standard method of phenol and isoamyl alcohol extraction, followed by ethanol precipitation²⁴. DNAs (10 μ g) were digested with *EcoRI* (Boehringer Mannheim) using conditions recommended by the supplier. Digested genomic DNA was separated by gel electrophoresis in 0.8% agarose, and blots were made onto nitrocellulose membrane²⁵. The recombinant plasmid pNB-1 (*N-myc*)⁵ was labelled with ³²P-dCTP by nick-translation²⁶ to a specific activity of 3×10^8 c.p.m. μ g⁻¹. Washing of filters after probing was carried out in either high-stringency conditions (0.1 $\times$ SSC, 0.2% SDS, 65 °C) (a) or conditions of moderate stringency (1 $\times$ SSC, 0.1% SDS, 65 °C) (b, c). The blots were reprobed with pESD14.1.1 (ref. 18) to control for the amount of DNA loaded per lane, and then transferred to the filter. The resultant ratios between the two signals were compared for all lanes except RB375A, RB475D, RB458 and RB447; the average ratio was *N-myc*/ESD = 0.84:1.0, with a range of 0.21–2.00, indicative of no amplification. a, RB355-7 is a subclone of RB355. This filter was washed to show the relative copy numbers of *N-myc*. Fibroblast DNA was used as a diploid control. b, c, Several RB samples and one osteogenic sarcoma (OS234). IMR32 and RB355 are positive controls for *N-myc*-amplification; fibroblast DNA is a diploid control. DNA sources are as follows (1) Fresh tumour material: RB475-A and -D (from separate eyes of one patient), RB493, RB458, RB457 and RB328. (2) Tumour cell culture: OS234, RB383, RB429R, RB405-A, RB430 and IMR32. (3) RB tumour cell culture derived from xenografts: RB355, RB355-7, RB412, RB409, RB247, RB447 and RB414.

Table 1 Relative levels of N-myc genomic amplification and mRNA in nine RB tumours, fetal retina, two OS tumours and control cell lines

Cells	Source	DM/HSR	DNA	Level of N-myc RNA	
				relative to β -tubulin	relative to ESD
Y79-T	C	+	40×	69×	4×
IMR32	C	+	20×	120×	NT
RB355	MC	+	35×	13×	NT
RB409	MC	-	1×	1.0×	1.0×
RB344	C	-	1×	1.2×	NT
RB247C	MC	-	1×	1.1×	NT
RG412	MC	-	1×	0.48×	NT
RB447	MC	-	1×	Present†	NT
RB430	C	-	1×	0.81×	NT
RB430	S	-	1×	NT	1.9×
RB517	S	-	1×	NT	1.9×
Fetal retina	S	NT	NT	0.32×	1.6×
Adult retina	S	NT	NT	NT	<0.05
Fetal brain	S	NT	NT	NT	0.27‡
Adult Brain	S	NT	NT	NT	<0.05
OS108	M	-	1×	NT	<0.05
OS234	M	+	1×	NT	<0.05
HL60	C	+	NT	<0.05	<0.05
HeLa	C	-	NT	<0.05	NT
Fibroblast	C	NT	1×	NT	NT

Tumour samples were obtained from three sources: S, surgical specimen; M, grow in nude mice; C, grown *in vitro*. Where there are two letters, the last letter indicates the source of tumour cells studied and the preceding letter indicates the culture history of the tumour cells; for example, MC indicates that the tumour was grown in xenografts in nude mice before passage to tissue culture. The presence of double minute (DM) or homogeneous staining regions (HSR) was tested on G-banded karyotype preparations. DNA copy number is given relative to single-copy fibroblast hybridization on Southern blots (Fig. 1). Relative levels of DNA and RNA were estimated by grain density using a BioRad 620 video densitometer and BioRad Model 3392A integrator. N-myc RNA levels were normalized with respect to β -tubulin¹⁷ or ESD¹⁸ signals. RB409 N-myc expression was assigned an arbitrary value of 1.0 as it was present on all Northern blots analysed. NT, not tested.

* An approximation because the signal was beyond the linear response region of the film.

† Densitometry was not done on this blot.

‡ Densitometry done using the longer exposure (see Fig. 2b).

expression, suggesting that regulatory mechanisms for N-myc are intact in these malignant cells. Genomic amplification of N-myc is restricted primarily to neuroectodermal tumours^{4,6-8,12,13}, and many correlate with poor clinical prognosis^{14,15}.

We studied genomic amplification of N-myc in 17 RB tumours (Fig. 1) and 2 OS cell lines derived from second primary malignancies in patients who had previously had RB (one OS tumour not shown). N-myc amplification was found in only one of 17 RB tumours, RB355¹², and in neither OS line despite cytological evidence of gene amplification. The known N-myc amplification in the neuroblastoma line IMR32⁵, and in the long-established RB line Y79^{7,16}, were used as positive controls. No rearrangements of N-myc were detected.

Nine RB tumours were examined for levels of N-myc expression using Northern blotting techniques. Seven RB tumours were studied after culture or xenografts, one directly from the surgical specimen (RB517) and one from both the surgical specimen and tissue culture (RB430). RB517 has a constitutional deletion at chromosome band 13q14. The presence of intact RNA on the Northern blots was determined by probing sequentially with N-myc and β -tubulin¹⁷ or concurrently with N-myc and a complementary DNA clone of the esterase D (ESD) gene¹⁸ (Fig. 2). ESD and β -tubulin are constitutively expressed¹⁹. All RB tumours expressed the expected major 4.0-kilobase (kb) transcript of the N-myc gene⁵. Fresh RB tumours expressed levels of the N-myc transcript comparable to those

in RB samples from cell lines or xenografts and fetal retina (Fig. 2; Table 1). RB430 was examined both as a fresh surgical specimen and after tissue culture for 3 years, and no change in N-myc expression was observed. Both RB tumours with genomic amplification, Y79 and RB355, and the neuroblastoma line IMR32, expressed N-myc at high levels. Expression of N-myc was not detected in HeLa cells or in the promyelocytic leukaemia cell line HL60. No N-myc signal was observed even when HL60 was overloaded in the Northern blot.

The cells of origin of RB have not yet been identified, but are probably the embryonic cells of the fetal retina²⁰. Therefore, we collected fetal retinas at weeks 6-10 of gestation, and pooled them for use as a source of RNA. We found that N-myc was expressed at equivalent levels in fetal retina, in the RB tumours without genomic amplification of N-myc, and in fresh RB tumours (Table 1). We suggest that the presence of N-myc messenger RNA in RB tumours is a consequence of the normal expression of this gene in the retinal precursor cells which transform into RB tumours. It is difficult to determine whether there is cellular heterogeneity in the specimens of fetal retina used since, morphologically, all of the cells in the retina have a similar appearance at this stage of development. The observation that the level of N-myc expression in fetal retina was equivalent to the level in pure populations of RB tumour cells is consistent with the conclusion that the fetal retinal specimen was not significantly contaminated with other cells. In fact, most retinal cells at this early developmental time may be potential precursors for RB tumour development. Alternatively, at this gestational stage all retinal cells, whether potential retinoblastoma precursors or not, may express N-myc transcripts. The lack of N-myc transcripts in adult retina indicates that N-myc expression is regulated during the differentiation of the retina and that the terminally differentiated cells no longer express the gene. Loss of the normal alleles at the *Rb-1* locus may prevent the normal differentiation of the fetal retinal cell involved in transformation, and thus N-myc expression may be maintained at embryonic levels of expression.

Fetal brain showed a low level of N-myc expression when the Northern blots were exposed more intensely (Fig. 2b). This low level is not surprising in view of the reported N-myc heterogeneity observed in fetal brain in early development²¹. On the Northern blot shown in Fig. 2, the adult brain sample was underloaded but did not give a signal for N-myc even in the more intense exposure, when control ESD transcripts were detectable (Fig. 2b). We conclude that N-myc expression is regulated during development of the brain, as has been shown in mouse brain²¹.

Patients with bilateral RB tumours are reported to have a predisposition to develop specific second malignancies, most commonly osteosarcomas, subsequent to and independent of the initial RB tumour²². Reduction to homozygosity in the region of the *Rb-1* locus also occurs in osteogenic sarcoma, indicating that recessive mutations of this gene are involved in the induction of osteosarcoma and retinoblastoma³. OS234 and OS108 are second tumours from RB patients; OS108 has been shown to reduce to homozygosity at chromosome 13³. The critical test for an intimate relationship between N-myc and the *Rb-1* mutation is the expression status of N-myc in the osteosarcomas of RB patients. Neither OS tumour studied was expressing N-myc (Fig. 2). Therefore, we conclude that *Rb-1* mutations can lead to malignancy without the overexpression or deregulation of N-myc.

Two other groups have studied N-myc expression in RB tumours. Lee *et al.*⁸ compared N-myc RNA levels in RB tumours with those in one human fetal retina of undefined gestational age and in one bovine adult retina. They detected N-myc expression only in the tumours, and concluded that N-myc was abnormally highly expressed in RB tumours and suggested that this gene could be the regulatory target of the RB gene. Schwab *et al.*⁶ detected no N-myc expression in three cell lines derived

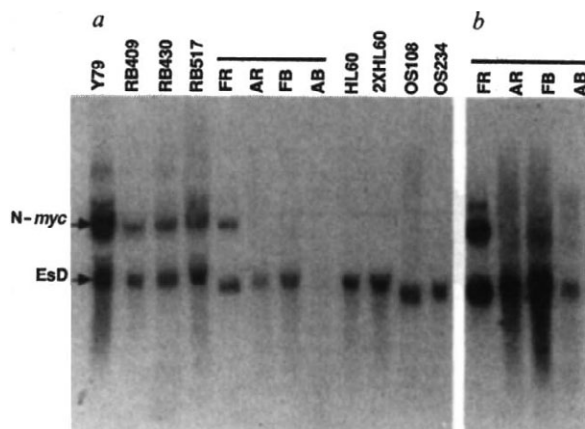


Fig. 2 Northern blot of two RB tumour cell lines (Y79, RB409), two fresh RB tumours (RB430, RB517), fetal (F) and adult (A) retina (R) and brain (B), two OS tumours (OS108 and OS234), and one promyelocytic leukaemia cell line (HL60). Total cellular RNA was prepared by guanidine thiocyanate-caesium chloride gradient centrifugation²⁷. Poly(A)⁺ RNA was obtained by oligo(dT)-cellulose chromatography. N-myc and pESD14.1.1 (ref. 18) were radiolabelled as described in Fig. 1 legend and simultaneously hybridized to GeneScreen membrane according to the method of Church and Gilbert²⁸. ESD served as an internal control for the presence of intact mRNA. 25 µg of total RNA was loaded per lane, except for three lanes: 50 µg of total RNA for 2 × HL-60; and 2 µg of poly(A)⁺ RNA for fetal retina and for OS108. *a*, 22-h exposure at room temperature with no intensifier; *b*, 19-h exposure at -70 °C with an intensifier.

from RB surgical specimens, but several observations in our laboratory suggest that these lines may have originated from the non-RB component of the surgical specimen: two of the cell lines do not have any of the cytogenetic abnormalities characteristic of RB tumours²³, fail to stain with a monoclonal antibody that reacts positively with all other RB tumours and lines, and do not resemble RB tumours in suspension tissue culture.

The expression of N-myc RNA in normal fetal retina and in RB tumours suggests that N-myc is a normal product in developing retina. The RB cell lines that do develop genomic amplification of N-myc may gain proliferative advantage by means of the accompanying excessive expression of N-myc, a gene normally functioning in the cells from which RB tumours arise. It is possible that N-myc is not normally expressed in the bone cells in which homozygosity for the RB mutation leads to OS tumours. Consequently, no N-myc transcripts are observed in the OS tumours. We have clearly demonstrated equal levels of expression of N-myc in RB tumours and in normal fetal retina, and that N-myc is not expressed in the OS tumours induced by the RB mutation. Thus, expression of N-myc cannot be essential to the initiating malignant process in these tumours.

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Discrete *cis*-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone genes

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The anterior pituitary gland, which is derived from a common primordium originating in Rathke's pouch, contains phenotypically distinct cell types, each of which express discrete trophic hormones: adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), prolactin, growth hormone, and follicle stimulating hormone (FSH)/luteinizing hormone (LH) (reviewed in ref. 1). The structurally related prolactin and growth hormone genes, which are evolutionarily derived from a single primordial gene², are expressed in discrete cell types—lactotrophs and somatotrophs, respectively—with their expression virtually limited to the pituitary gland¹. The pituitary hormones exhibit a temporal pattern of developmental expression with rat growth hormone and prolactin characteristically being the last hormones expressed³⁻⁸. The reported co-expression of these two structurally related neuroendocrine genes within single cells prior to the appearance of mature lactotrophs, in a subpopulation of mature anterior pituitary cells, and in many pituitary adenomas^{1,6-9} raises the possibility that the prolactin and growth hormone genes are developmentally controlled by a common factor(s). We now report the identification and characterization of nucleotide sequences in the 5'-flanking regions of the rat prolactin and growth hormone genes, respectively, which act in a position- and orientation-independent fashion to transfer cell-specific expression to heterologous genes. At least one putative *trans*-acting factor required for the growth hormone genomic sequence to exert its effects is apparently different from those modulating the corresponding enhancer element(s) of the prolactin gene because a pituitary 'lactotroph' cell line producing prolactin but not growth hormone selectively fails to express fusion genes containing the growth hormone enhancer sequence.

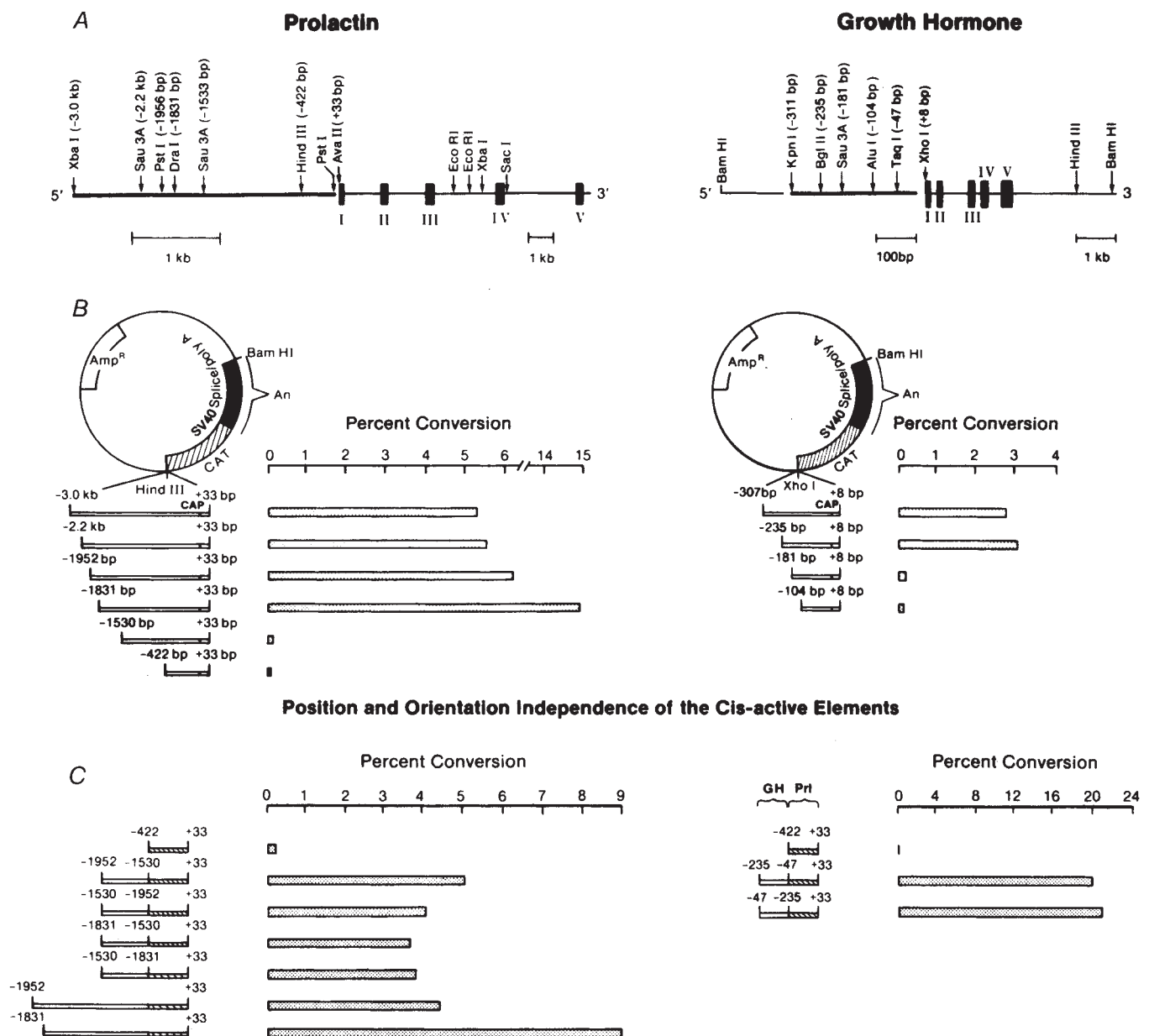


Fig. 1 Identification of *cis*-active region(s) in the 5' flanking prolactin and GH genomic sequences which confer cell-specific expression. **A**, Activity of rat prolactin genomic, CAT fusion constructions. The sites used for the constructions are listed on the map of the rat prolactin and growth hormone genes, based on previous reports^{2,18-20}. Fragments of the indicated sizes, each utilizing a common 3' end (+33 for prolactin, +8 for growth hormone) were ligated into pSV2 CAT such that the promoter and CAP site were contributed by the prolactin or growth hormone genomic fragments. No viral enhancers were present in the final construction. **B**, Sites used for deletion analysis are indicated, and the diagrammed fusion genes were transfected into rat pituitary cell lines (GH₄, G/C). Mapping the *cis*-active genomic sequences for each gene was accomplished using expression of a reporter gene, bacterial CAT¹⁵, in GH₄ cell lines, assessed in cells harvested 48 h after transfection. Results are the average of duplicate determinations differing by <5% of 3–12 experiments in GH₄, G/C, and GH₃ cell lines. All data were significant ($P < 0.001$). **C**, Position- and orientation-independence of the rat prolactin and GH genomic enhancer sequences. A 188-bp GH genomic enhancer sequence (–235 to –47) was placed in both orientations 5' to a gene containing the prolactin promoter fused to the CAT gene (the *Prl*-422 CAT construction) and used to transfect GH₄ cells in three experiments of similar design. Prolactin genomic fragments (–1,831 to –1,532) and (–1,952 to –1,532) were fused in both orientations to the *Prl*-422 CAT construction. Transfections were performed in GH₄ cells. In additional experiments, the –235 to –47 and –1,831 to –1,532 regions of the growth hormone and prolactin genes, respectively, were placed in both possible orientations 3' of the CAT transcription unit, located approximately 350 bp 3' of the poly(A) site in the *Prl*-422 CAT construction. Even in the inverted (3' to 5') orientation, these constructions were each expressed 75–80% as efficiently as the constructions shown above in which the identical genomic fragments were placed 5' of the prolactin gene promoter. Placing the identical *cis*-active sequences of the prolactin or GH lines immediately 5' of a herpes *tk* promoter, CAT gene fusion converted the inactive transcription units (0% conversion) to an active unit (for example 19% and 15% conversion, respectively) in GC₂ cells; similar results were obtained using constructions containing the RSV promoter. These elements exerted no enhancement of herpes *tk* promoter, CAT, or RSV promoter, CAT fusion gene expression in 208 F fibroblast cells. Results were confirmed in three to five experiments of similar design.

Methods. The fusion genes containing the GH fragments diagrammed above were constructed by converting the HindIII site in pSV2 CAT to an XhoI site and ligating GH 5' flanking fragments into the BamHI, XhoI sites of the vector. 5' GH sites were converted to BamHI sites following filling using T4 polymerase for the KpnI site, or ligating into the BamHI in the case of BglII and SauIIIA sites, using conventional techniques, and placing these fragments into pSV2 CAT, as diagrammed. In the case of the *prl*/CAT fusion genes, an AvaII site 33 nucleotides 3' to the CAP site was converted to a HindIII site and indicated 5' sites (XbaI, PstI, DraI, SauIIIA) were either converted to BamHI sites using T4 polymerase-catalysed fill reactions where appropriate or ligated into the BamHI site (SauIIIA). The vector containing the HindIII to AvaII (–422 to +33) fragment of the prolactin gene fused to the CAT gene is referred to as *Prl*-422 CAT. In addition to the diagrammed constructions, a 5' flanking GH sequence extending –7 kb (using an EcoRI site) and an XhoI to BamHI fragment encompassing the entire coding portion and 2.7 kb of 3' flanking GH genomic sequences ligated in opposite orientation 5' to the *Prl*-422 CAT fusion gene were tested; these constructions did not exhibit enhancer function.

To ascertain the existence of genomic regions that determine pituitary-specific gene expression, fusion genes containing 3 kilobases (kb) or 235 base pairs (bp) of prolactin or growth hormone genomic 5'-flanking sequences, respectively, were fused to a reporter gene encoding bacterial chloramphenicol acetyltransferase (CAT) (Fig. 1) and introduced into a series of heterologous cell lines. As shown in Table 1, the fusion genes are expressed only in prolactin and growth hormone-producing pituitary cell lines (GH₄, G/C).

The growth hormone and prolactin *cis*-active elements responsible for cell-specific expression were characterized by generating a series of deletions extending from -3 kb to -172 bp in the 5' flanking prolactin genomic sequences (Fig. 1A) and from -2 kb to -104 bp of the 5' flanking growth hormone genomic sequences (Fig. 1A). In the case of the prolactin genomic sequences, enhanced expression of the CAT gene product was observed with sequences extending 1.83 kb 5' of the prolactin gene transcription initiation (CAP) site, and was virtually eliminated with deletion to -1.53 kb, although a small residual enhancement was observed with 5' flanking fragments as short as 172, but not 70, base pairs. In the case of growth hormone 5' flanking genomic sequences, maximally enhanced expression of the reporter gene was observed in constructions containing >235 base pairs of 5' flanking information, while deletion to -181 base pairs 5' of the CAP site virtually abolished expression of reporter function in these cells (Fig. 1B).

As shown in Fig. 1C, both prolactin and growth hormone genomic fragments extending from -1.95 or -1.83 to -1.53 kb and from -235 to -47, respectively, fused to a 422-bp prolactin gene promoter region resulted in marked stimulation of the expression of the fusion genes in the growth hormone-producing cells, irrespective of orientation or position. The prolactin or growth hormone *cis*-active elements enhanced reporter gene expression even when placed 3' of the reporter gene (Fig. 1G). Primer extension analysis confirmed correct CAP site usage in both the prolactin and growth hormone constructions (Fig. 2).

The prolactin and growth hormone *cis*-active sequences acted in a cell-specific manner independent of possible transcriptional restriction imposed by their respective promoters. Chimaeric genes containing either the prolactin genomic fragment from -1.83 kb to -1.53 kb or the growth hormone genomic fragment from -235 bp to -47 bp ligated to a herpes thymidine kinase (*tk*) promoter, CAT gene fusion resulted in no enhancement of gene expression in heterologous cell types, while expression of these chimaeric genes in GH₄ and G/C cell lines was markedly enhanced (Fig. 1C). The possibility that a restriction in tissue-specific expression was imposed by the prolactin promoter itself is unlikely because the prolactin promoter (-422 to +33) was efficiently expressed in fibroblasts and CV1 cells in the presence of the simian virus 40 (SV40) enhancer (Table 1). Despite the 2.5- to 3.5-fold increase in expression of constructions containing the -1.83 kb compared to -1.95 kb 5'-flanking prolactin genomic fragment (Fig. 1B), the presence of a biologically important suppressor or silencer sequence appears less likely because both the -1.95 to -1.53 and -1.83 to -1.53 kb fragments exerted comparable effects when inserted in front of the prolactin genomic promoter extending to -422 bp (Fig. 1C).

Based on these analyses, we conclude that a 298-bp sequence located approximately 1.85 kb upstream of the prolactin gene CAP site, and a 188-bp sequence located approximately 200 bp upstream of the growth hormone gene CAP site, act as enhancers in the GH cells and appear to be critical for the tissue-specific expression of these genes.

More detailed analyses were performed to identify the minimal sequences in the prolactin and growth hormone genes sufficient for enhancer function. As diagrammed in Fig. 3, a series of genomic fragments inserted in front of the prolactin promoter, CAT fusion gene (the -422 to +33 fragment, the plasmid referred to as *Prl*-422 CAT, Fig. 1) were tested for ability to confer enhanced expression of the reporter gene.

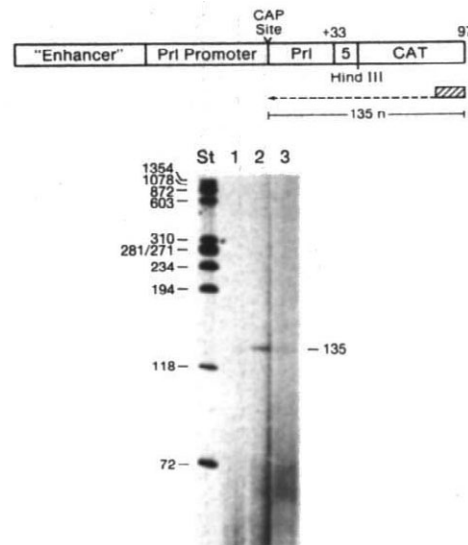


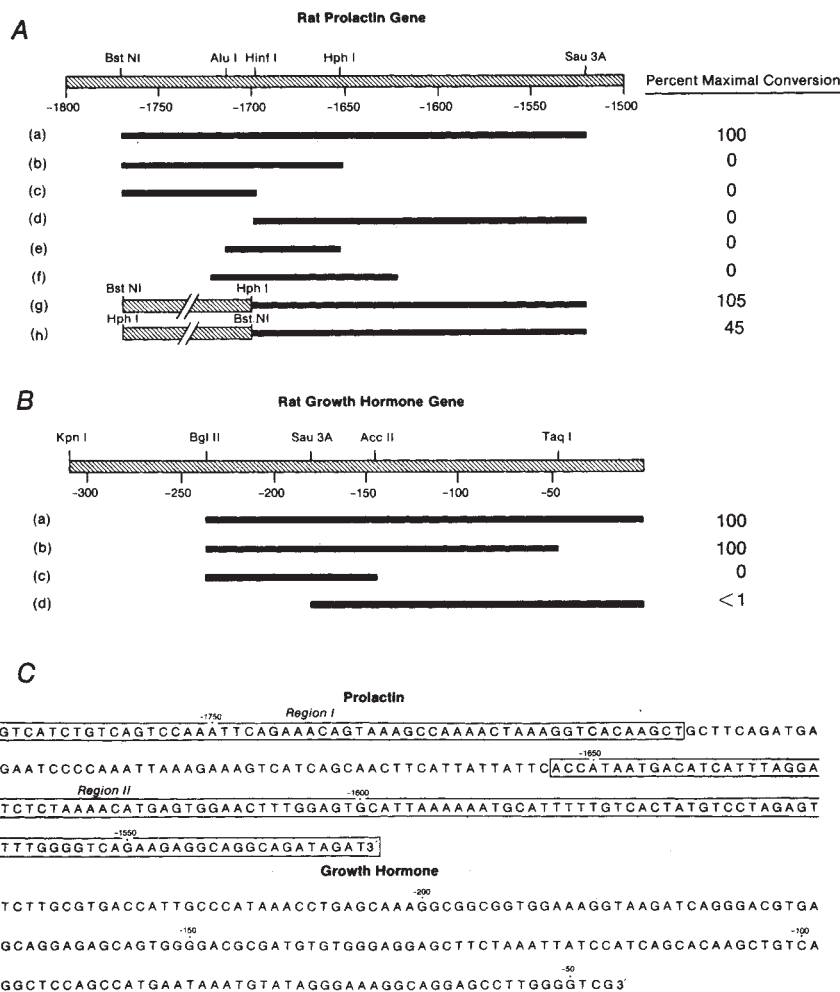
Fig. 2 Mapping the transcription initiation sites of the chimaeric genes containing the prolactin and growth hormone enhancer elements. Chimaeric genes containing the growth hormone or prolactin *cis*-active elements (-235 to -47 and -1,952 to -1,532, respectively), fused to the *Prl*-422 CAT fusion gene, as described in Fig. 1C, were transfected into GC₂ cells, and RNA was prepared and subjected to primer extension analysis, as described under methods. As diagrammed in A, for both constructions, the expected primer extension product was 135 nucleotides if the expected prolactin gene CAP site was utilized. **B**, The primer extension products using RNA from cells transfected with: lane 1, the prolactin -1,952 to -1,532, *Prl*-422 CAT fusion gene, from which the prolactin promoter region (-422 to -33) was removed. No CAT activity was detected in cells transfected with this construction; lane 2, the prolactin -1,952 to -1,532, *Prl*-422 CAT fusion gene; lane 3, the growth hormone -235 to -47, *Prl*-422 CAT fusion gene. Standards are the *Hae*III digests of Φ X174 DNA. Autoradiograph shown was developed following a 7-day exposure.

Methods. Total RNA was isolated from GC₂ cells 48 h following transfection with 10 μ g plasmid DNA per plate, by homogenization in thiocyanate and pelleting through cesium chloride to remove DNA. A single-stranded primer of sequence 5' GTTCTTTACGATGCCATTGGGATATATCAACGG 3' complementary to nucleotides 29-61 of the CAT coding sequence, was labelled on the 5'-terminus to a specific activity of 10^9 c.p.m. μ g⁻¹ using T₄ polynucleotide kinase and (γ -³²P)ATP (7,000 Ci mmol⁻¹, ICN). For each reaction, 50 μ g total RNA and 3 ng labelled primer were heated to 70 °C in 12 μ l RT buffer (50 mM Tris pH 8.0, 8 mM MgCl₂, 50 mM KCl, 40 mM dithiothreitol) and slowly cooled to 55 °C. The annealed primer and RNA were then precipitated with 0.3 M sodium acetate and 1 volume of isopropanol and resuspended in RT buffer. dATP, dCTP, dGTP and dTTP were added, to a final concentration of 1 mM each in a final volume of 20 μ l. The reaction was initiated with 24 units of reverse transcriptase and allowed to proceed for 30 min at 42 °C. After phenol/chloroform extraction, the extension products were analysed on 8% denaturing polyacrylamide gels.

Deletion of 61 5' nucleotides of the 298-bp prolactin enhancer element (-1,831 to -1,769) resulted in no loss of activity (Fig. 3 (a)); however, dividing the region by cleavage at a *Hinf*I site at nucleotide -1,698 resulted in virtually complete loss of activity of both of the resultant fragments (Fig. 3 (c), (d)), apparently splitting a critical site or separating two discrete sequences. To test the possibility that a critical site was split, regions extending both 5' and 3' of the *Hinf*I site were tested. Neither a 50-bp sequence (-1,713 to -1,663), nor a 98-bp fragment (-1,722 to -1,625) exhibited any enhancer activity (Fig. 3 (e), (f)). Therefore, a fragment containing 106 nucleotides including the 5' sequences known to be sufficient for activity (-1,769 to -1,663) was tested. This region was entirely silent in GH cell lines (Fig.

Fig. 3 Mapping the 5' and 3' limits of the *cis*-active prolactin and growth hormone genomic sequences specifying cell-specific gene expression. **A**, A series of prolactin 5' genomic restriction fragments were prepared as diagrammed and placed 5' to the fusion gene *Prl*-422 *CAT*, containing the prolactin genomic sequences from -422 to +33 (shown in Fig. 1). These plasmids were used to transfect GH₄, G/C, and 235-1, as described in the legend to Fig. 1. The -1,769 to -1,530 fragment, which gave a 24 ± 8% conversion in different experiments, represented 100% for the prolactin gene fragment series. Similar results were obtained in five experiments of similar design. The fragment -1,769 to -1,663 exhibited 0% of expression in five separate experiments (GH₄, G/C and 235-1 cell lines); but 15% maximal activity was observed in two separate experiments. **B**, Growth hormone genomic fragments were tested for their ability to direct *CAT* expression in GH₄ cells. The -235 to -47 fragment and the -235 to -146 fragment were fused to the prolactin -422 *CAT* fusion gene. The -235 to -47 fusion gene activity (5-12% conversion in separate experiments) represented maximal activity (100%). **C**, Sequences of the cell-specific enhancer elements of the rat prolactin and growth hormone genes. The boxed sequences indicate regions which are critical for activity based on deletion mapping in **A**. There appear to be two functionally separable regions in the prolactin enhancer (Region I and Region II). The growth hormone sequence is based on the data of Barta *et al.*²⁰. The prolactin genomic sequence was determined for the fragments used in these experiments using dideoxynucleotide, enzymatic sequencing methods²¹, and is in agreement with the recently published data of Maurer¹⁹, except for the identification of residue -1,667 as a T. Results shown are the average of three or four separate experiments.

Methods. *Prl* 5' flanking fragments from *Bst*NI, *Sau*III A (-1,769 to -1,530), *Bst*NI, *Hinf*I (-1,769 to -1,695), *Hinf*I, *Sau*III A (-1,697 to -1,530), *Alu*I-*Hph*I (-1,713 to -1,663 and *Bst*NI, *Hph*I (-1,769 to -1,663) were inserted into the plasmid *Prl*-422 *CAT* using the strategy described in the legend to Fig. 2. The 98-bp fragment extending from -1,722 to -1,625 was constructed by synthesis of two synthetic oligonucleotides using phosphoramidite chemistries (71-mers) corresponding to sense (-1,722 to -1,658) and antisense (-1,691 to -1,625), and including sequences generating a 5' *Xho*I site and 3' *Bam*HI site, annealing, filling using the Klenow fragment of *Escherichia coli* polymerase, cleaving with *Xho*I and *Bam*HI, and inserting into the *Prl*-422 *CAT* vector. The final construction involved placing the *Bst*NI to *Hph*I fragment (-1,769 to -1,663) ligated with *Xho*I linkers, in front of the construction containing the *Hinf*I to *Sau*III A fragment (-1,697 to -1,530). All plasmids were purified by two CsCl gradients prior to transfection, which was performed using GH₄, G/C, and 235-1 cell lines. Results are the average of duplicate determinations (50 µg protein, 10 h assays) in three separate experiments. The *GH* genomic constructions involved ligating an *Xho*I linker at the *Acc*II site, and inserting the resultant *Bgl*II, *Xho*I fragment (-235 to -146) into the *Prl*-422 *CAT* construction. Other constructions are described in the legend to Fig. 1. Transfections and analysis of *CAT* activity are described for above.



3 (b)); however, in two experiments with GC₂ cells, approximately 15% of maximal activity was observed, perhaps reflecting altered levels of a required *trans*-acting factor(s) in some GH cell lines. Based on these data, it appeared possible that there were two separate regions required for prolactin gene enhancer function. This possibility was tested by the ligation in both possible orientations of the inactive 106 nucleotide fragment (-1,769 to -1,663), to the silent region 3' of the *Hinf*I site (-1,697 to -1,530) (see Fig. 3 (g), (h)). Although in five separate experiments neither component fragment alone exhibited any activity, the chimaeric constructions were always active. The fusions in which the two fragments were in the same orientation exhibited 100% of maximal enhancer activity (Fig. 3 (g)), while the chimaeric construction in which the 106 nucleotide 5' fragment was placed in a 3' to 5' orientation exhibited approximately 45% of maximal enhancer activity (Fig. 3 (h)). Inspection of the fusion junctions revealed no fortuitous reconstitution of sequences around the *Hinf*I or *Hph*I sites in either construction.

These data indicate the presence of two discrete, separable sequences, both of which are required for full enhancer function, with no absolute constraints on the relative position or orientation of these two elements for their functional complementation.

Because an 89-bp growth hormone gene region extending from -235 to -146 was insufficient to produce any enhancement, there would appear to be >35 bp spanning the 5' and 3' boundaries of the growth hormone gene enhancer sequence (Fig. 3).

To test the possibility that different factor(s) confer activity characteristic of the respective enhancer elements, the prolactin and growth hormone genes were tested in a rat pituitary cell line (235-1) which produces prolactin but no detectable growth hormone¹⁰. Although the prolactin enhancer is highly active in this cell line, the growth hormone enhancer fails to function even when fused to the prolactin gene promoter (Fig. 4), suggesting different *trans*-acting factors regulate the two enhancers despite the evolutionary relatedness of the two genes. A chimaeric construction containing the prolactin enhancer

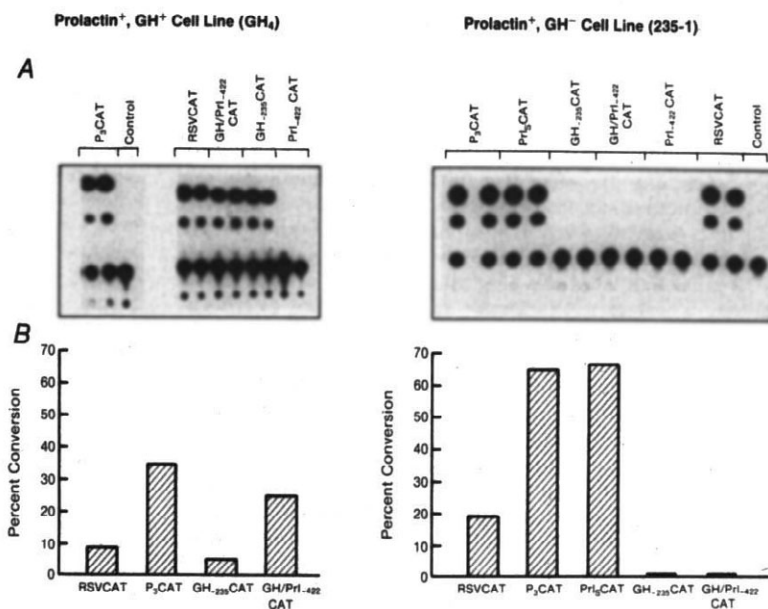
Table 1 Identification of *cis*-active regions in the 5' flanking sequences of the rat prolactin and growth hormone genes

Cell	Species	Type	Per cent conversion		
			<i>Prl</i> -5'F	<i>GH</i> -5'F	RSV-EN
MCF7	Human	Mammary epithelium	0	0	18
208F	Rat	Fibroblast	0	0	70
F9	Mouse	Embryonic	0	0	50
HIT	Hamster	Pancreatic (endocrine)	0	0	19
MTC-CA	Rat	Thyroid 'C' cell (endocrine)	0	0	6
JEDEDIAH	Rat	Pituitary (non-endocrine)	0	0	28
GH ₄	Rat	Pituitary (endocrine)	6	4	22
G/C	Rat	Pituitary (endocrine)	11	15	15

Fragments extending from -3 kb to +33 bp of the rat prolactin (*Prl*) gene and from -235 to +8 bp of the rat growth hormone (*GH*) gene were fused to the *Hind*III site of the bacterial chloramphenicol acetyl transferase (*CAT*) gene¹⁵, as diagrammed in Fig. 1, such that the promoter and cap site were contributed by the inserted fragment (referred to as *Prl* 5'F and *GH* 5'F, respectively). These constructions, and a comparable construction containing the Rous sarcoma virus (RSV) promoter/enhancer [RSV-EN] were used for DNA mediated gene transfer using a series of cell lines, and the activity of expressed *CAT* enzyme was quantitated 48 h later; the per cent conversion of ¹⁴C-chloramphenicol to the acetylated form is presented. Results are the average of duplicate determinations differing by <5%, and the results were confirmed for each cell line in 3-15 separate experiments. The average expression $\pm$ s.e.m. for the prolactin and growth hormone fusion genes was $18.2\% \pm 3.5\%$ and $4.3\% \pm 0.8\%$, respectively; results were significant at $P < 0.001$. In other experiments, RSV *CAT* recorded up to 45% conversion in MTC-CA cells while *Prl* 5'F and *GH* 5'F still gave undetectable signals. SV40 enhancer, promoter and Moloney leukaemia virus (MLV) enhancer, promoter, *CAT* fusions were also used as controls, confirming efficient transfection into the heterologous cell lines. Function of the prolactin promoter in heterologous cell lines was confirmed by demonstration of reproducibly efficient expression of chimaeric constructions containing SV40 enhancer, prolactin promoter (*Prl*-422, *CAT*, Fig. 1) in 208F fibroblasts and CV1 cells (6-8% and 25-30% conversion, respectively). A construction containing the MLV enhancer, prolactin promoter was also expressed in these cell lines. 0 = no detectable conversion above mock transfection.

Methods. Plasmids were constructed as described in the legend to Fig. 1, using a *Bgl*II-*Taq*I (-235 to +8 bp) fragment of the *GH* gene; and *Xba*I site (-3 kb) and *Ava*II sites (+33 bp) of the prolactin gene, and the *Bam*HI and *Hind*III sites of the vector following appropriate site conversions using synthetic linkers by standard techniques. *Xho*I and *Hind*III linkers were added at the *Xba*I and *Ava*II sites, respectively, of the prolactin genomic fragment, and the fragment was ligated into the *Xho*I/*Hind*III sites of the modified vector pSV2 *CAT*¹⁵. The *Hind*III site of pSV2 *CAT* was converted to an *Xho*I site prior to the insertion of the *GH* fragments into the *Bam*HI/*Xho*I sites. All transfections were performed using 10 μ g ml⁻¹ of plasmid DNA and the calcium phosphate co-precipitation method¹⁶ or DEAE-dextran sulphate method¹⁷ 48 h prior to harvest. Chloramphenicol acetyl transferase activity was quantitated using 50 μ g of protein extract and 10 h incubations, and subjected to chromatography as previously described¹⁵. The RSV, *CAT* plasmid construction used has been reported¹⁷. In the assay conditions used, there was a linear relationship between chloramphenicol acetylation and *CAT* activity. Each cell line was grown in serum-containing medium, and medium was changed 3 h prior to transfection.

Fig. 4 Evidence for discrete *trans*-acting factors mediating the prolactin and growth hormone enhancers. A rat pituitary cell line of lactotroph origin (235-1)¹⁰, which produces only prolactin was used to assess cell-specific action of the *GH* and *Prl* gene enhancer sequences. Plasmids analyzed were the *GH* (-235 to +8) *CAT* fusion (*GH*-235 *CAT*), the RSV *CAT* fusion (RSV *CAT*), the *Prl* (-422 to +33) *CAT* fusion (*Prl*-422 *CAT*), and a *GH* (-235 to -47) fragment inserted into the *Prl*/*CAT* fusion gene (*GH*/*Prl*-422 *CAT*), the 3-kb prolactin *CAT* fusion (*P3* *CAT*), and a fusion gene containing *Prl* -1,831 to -1,530 ligated to the *Prl*-422 *CAT* (*Prl*_s *CAT*), as diagrammed in Figs 1 and 2. A shows the autoradiograph of the *CAT* assay of the plasmids transfected into GH₄ cells (left) or 235-1 cells (right). B shows the per cent conversion for both cell lines in a different experiment. Results were confirmed in five separate experiments of similar design, using 50 μ g protein, 10 h assays as previously described.



(-1,831 to -1,530) fused 5' to the 235 bp growth hormone flanking region was analysed for expression in the 235-1 cell line and found to be fully active, indicating the growth hormone promoter region was competent for expression, and that failure to express the growth hormone gene in 235-1 cells reflects the apparent absence of a critical *trans*-acting factor.

Despite their reported co-expression during development⁸, the cell-specific expression of the growth hormone and prolactin genes appears to be controlled by structurally distinct, *cis*-active sequences under regulation of discrete *trans*-acting factors. The

location of the prolactin gene enhancer described in this manuscript corresponds to a reported pituitary-specific pattern of chromosomal DNase I hypersensitivity, approximately 1.8 kb upstream of the CAP site¹¹. Because the prolactin *cis*-active sequence contains two separable regions which can be placed in either orientation with respect to each other and continue to functionally complement, we suggest that two (or more) *trans*-acting factors are required for the prolactin gene enhancer function in lactotrophs. Exonuclease protection assays of the prolactin enhancer region following binding of extracts from

GH cells tentatively identify at least two distinct and specific protein binding regions coincident with those defined by the mapping analyses.

Although the enhancer sequence of the growth hormone gene contains a region (at -190 bp) which corresponds to the 'core' enhancer sequences identified in the immunoglobulin and several viral genes¹²⁻¹⁴, the growth hormone enhancer core sequence alone is insufficient for function because a region containing >40 bp on each side of this sequence exhibits no enhancer activity. Preliminary footprint analysis reveals that one region located 3' in the growth hormone gene enhancer specifically binds a factor in cells which express the gene (GH cells) but not in cells (235-1) in which the growth hormone enhancer fails to function.

Further understanding of the molecular basis for the selective activation or/and silencing of growth hormone or prolactin gene expression in the development of mature somatotrophs and lactotrophs will require analysis of the expression of transcription units containing heterologous promoters and the respective enhancer sequences in transgenic animals, and the identification and characterization of the cognate *trans*-acting factors.

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Transcriptional interference and termination between duplicated α -globin gene constructs suggests a novel mechanism for gene regulation

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The interesting possibility that transcriptional interference can occur between eukaryotic genes was raised by studies on the avian leukosis retrovirus (ALV)¹ which showed that deletion of the promoter in the 5' long terminal repeat (LTR) activates the 3' LTR promoter, linked to a downstream gene. These observations provide a molecular explanation for the fact that insertional oncogenesis by the ALV promoter is invariably associated with either a rearranged or deleted 5' LTR sequence²⁻⁴. This letter extends these findings to chromosomal RNA polymerase II genes by studying transcriptional interference between duplicated α -globin gene constructions. I demonstrate that transcriptional interference causes substantial inhibition of the downstream α gene by transcription of the upstream α gene. Furthermore, this inhibition is alleviated by placing transcriptional termination signals between the two α genes. Because many eukaryotic genes may be arranged in tandem on a chromosome, these observations suggest that transcriptional termination is an important mechanism for preventing interference between adjacent genes. The selective use of termination signals may provide a novel way of regulating the activity of eukaryotic genes.

The HeLa cell transient expression procedure^{5,6} was used initially as a convenient system to study α -globin gene transcription. In this technique, the α genes are introduced into HeLa cells as part of an episomal plasmid that may be present at copy numbers as high as 10,000 or more per cell nucleus⁶. Figure 1a shows a diagram of the pSVod plasmid⁶ containing two human α -globin genes ($\alpha\alpha$ pSVod). The α genes are separated by about 700 base pairs (bp) and each one contains more than 300 bp of 5'-flanking sequences but only about 100 bp of 3'-flanking

sequence. Therefore, although the poly(A) sites of each α gene are intact, they are unlikely to contain possible 3'-flanking region termination signals. pSVod is a transient expression vector based on pBR322 containing the simian virus 40 (SV40) replication origin but not the enhancer sequence (72-bp repeat). The SV40 early and late promoters are also present in the SV40 origin (ori) region but will be largely inactive in pSVod, since the early promoter requires the enhancer^{7,8}, and the late promoter has its major initiation sites within the 72-bp repeat sequence, absent in pSVod⁹. Co-transfection of pSVod with another plasmid (SVpBR328R β)¹⁰ containing both the SV40 large-T antigen and rabbit β -globin genes allows replication of SV40 origin-containing plasmids⁵ as well as providing a control to test for efficiency of transfection. The 5' α -globin gene has a 13-bp insert in its 5' noncoding sequence, called Tag, to allow the otherwise identical 5' termini of the two α -gene transcripts to be distinguished by the primer extension RNA mapping technique¹¹. Figure 1a also shows two constructs in which either the 5' α -gene or 3' α -gene promoter is deleted (Δ P $\alpha\alpha$ and $\alpha\Delta$ P α).

Figure 1b shows the primer extension analysis on $\alpha\alpha$, Δ P $\alpha\alpha$ and $\alpha\Delta$ P α transfections. The relative efficiency of each transfection is indicated by the intensity of the 3'-end signal produced by S₁ nuclease mapping¹² of rabbit β -globin messenger RNA in turn produced by the co-transfected plasmid shown under each lane. RNA from cells transfected with $\alpha\alpha$ pSVod gives two extension products corresponding to the Tag-containing 5' α -gene mRNA and normal 3' α -gene mRNA. The product bands are doublets, as is usually observed with primer extension, possibly reflecting partial extension onto the 5' CAP structure of the mRNA. Deletion of the promoter of the 5' α gene as shown by Δ P $\alpha\alpha$ results in an approximately threefold increase in the 3' α -gene signal. Surprisingly, deletion of the promoter of the 3' α gene also results in an approximately threefold increase in the 5' α -gene signal as can be seen by comparing $\alpha\alpha$ and $\alpha\Delta$ P α . These data indicate that each α -gene promoter exerts an inhibitory effect on the other α -gene promoter. One possible cause of this inhibition could be through a competitive effect between the two α -gene promoters for possibly limiting factors. Alternatively, a transcriptional interference mechanism may be responsible for the observed modulation in mRNA levels.

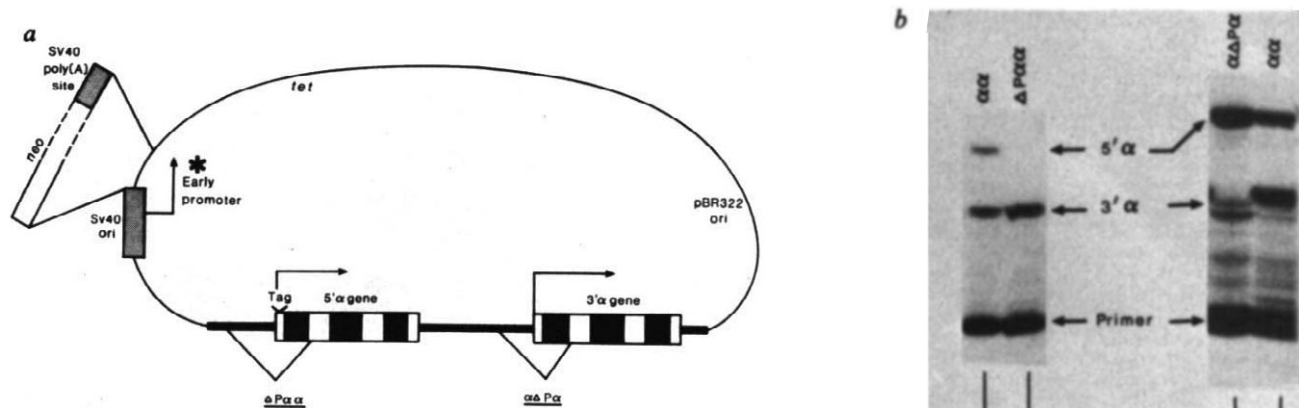


Fig. 1 **a**, Diagram of α pSVod (ref. 6). α Genes are depicted by a thick line for flanking sequences, open boxes for noncoding and intron sequences, solid boxes for exon sequences. The positions of the promoter deletions in $\Delta P\alpha$ and $\alpha\Delta P\alpha$ are indicated. $\leftarrow$ Denotes position and direction of promoter. * Indicates that the SV40 early promoter is only functional in pSVedneo. The pBR322 sequence is depicted by a thin line. Note that the sequence in pBR322 found to inhibit SV40 replication (between the pBR322 replication origin and tetracycline resistance gene (*tet*) gene) is deleted in pSVod. SV40 sequences are depicted by shaded boxes. The SV40 ori sequence in pSVod contains the replication origin but not the enhancer. As indicated, the neomycin resistance gene and SV40 poly(A) site¹⁷ were added to the plasmid. In addition, the SV40 origin region was expanded slightly to include the enhancer sequence as this is required for the early promoter^{7,8}. **b**, Primer extension RNA analysis¹¹ on cytoplasmic RNA purified from HeLa cells transiently transfected with α -; $\Delta P\alpha$ - and $\alpha\Delta P\alpha$ pSVod, together with the co-transfection control plasmid SVpBR328R β 1 (ref. 10). 3'-End S₁ mapping¹² data on rabbit β -globin mRNA produced by SVpBR328R β 1 are shown beneath each lane.

Methods. DNA constructions: The Tag α promoter was constructed by ligating a 134-bp human α -globin gene *Hinf*I fragment containing 112 bp of 5'-flanking sequence together with 22 bp of 5' noncoding sequence into the polylinker *Sall* site of pUC9²². This *Hinf*I fragment contained all the sequence required for initiation of transcription of the α 1-globin gene promoter⁶. Next, an 894-bp *Hinf*I-*Pst*I fragment containing the rest of the α 1-globin gene was ligated into the *Bam*HI site of pUC9, next to the previously used *Sall* site. This effectively results in expansion of the 5' noncoding *Hinf*I site of the α 1-globin gene by 13 nucleotides and thereby 'Tags' the 5' end of the α 1 gene²³. α -, $\Delta P\alpha$ - and $\alpha\Delta P\alpha$ pSVod: Tag and $\Delta P\alpha$ pSVod were made by cutting α 1pSVod⁶ with *Sma*I to remove the promoter region and exon 1 of α 1. Tag α pSVod was then made by ligating the same *Sma*I fragment from Tag α 1pUC9 into the *Sma*-cut α 1pSVod vector. $\Delta P\alpha$ pSVod was made by ligating α 1pSVod *Sma* vector back together without an insert fragment. The second α 1 gene as a 1.6-kb *Pst*I fragment was then added to Tag or $\Delta P\alpha$ 1pSVod at a unique *Pst*I site in the 3'-flanking sequence of the α gene to make $\alpha\alpha$ pSVod and $\Delta P\alpha\alpha$ pSVod. Similarly, a $\Delta P\alpha$ 1 gene fragment was added 3' to Tag α 1pSVod to make $\alpha\Delta P\alpha$ pSVod. pSVedneo: The plasmid pSV2*neo containing the Tn5 neomycin phosphotransferase gene which contains the SV40 early promoter and poly(A) site flanking the *neo* gene has been described elsewhere¹⁷. A *Hind*III-*Bam*HI fragment containing the *neo* gene and the SV40 early poly(A) site from pSV2*neo was ligated into pSVed between the *Hind*III and *Bam*HI sites. pSVed is a variant on pSVod containing slightly more SV40 origin sequence to include the transcriptional enhancer²⁴. Various α -gene-containing pSVod plasmids were then converted into pSVedneo plasmids by exchanging the *Eco*RI-*Bam*HI fragment in pSVod for the equivalent SV40 origin *neo* fragment in pSVedneo. **HeLa cell transient expression:** Transfections were carried out as described previously^{6,24}. Briefly plasmid DNA was precipitated with calcium phosphate and added to subconfluent Petri dishes of HeLa cells. After 10–16 h, the medium was changed and the cells allowed to grow for another 48 h. The HeLa cells were collected, lysed in Nonidet P-40 detergent buffer, and the cytoplasmic and nuclear fractions separated by centrifugation through a sucrose cushion. Following incubation with proteinase K, cytoplasmic RNA was purified by phenol chloroform extraction and ethanol precipitation. **RNA mapping:** Primer extension¹¹: The DNA primer for α -globin mRNA 5'-end analysis was a single-strand *Hinf*I-*Hae*III fragment extending from the middle of the first exon to the middle of the 5'-noncoding region of the α 1-globin gene. This was obtained by filling in the *Hinf*I end of the double-strand DNA with [α -³²P]dATP and fractionating on a denaturing 7 M urea 12% polyacrylamide gel. DNA primer (20 c.p.m., specific activity 3,000 Ci mmol⁻¹) and cytoplasmic RNA (~20 μ g) were annealed in 10 μ l of 10 mM PIPES pH 6.4, 0.4 M NaCl at 80 °C for 10 min and at 63 °C overnight. 50 μ l of reverse transcriptase buffer (50 mM Tris pH 8.2, 10 mM dithiothreitol, 6 mM MgCl₂, 0.5 mM dATP, dCTP, dTTP and dGTP plus reverse transcriptase (5 U)) were added to hybridizations and incubated at 42 °C for 1 h. RNase (20 μ g) was added to incubation and left a further 15 min at 42 °C. The reaction was phenol extracted, ethanol precipitated and fractionated by electrophoresis on 7 M urea polyacrylamide gels. S₁ mapping¹²: SVpBR328R β 1 was digested with *Eco*RI and the ends filled in with [α -³²P]dATP using standard procedures. This gives a rabbit β -globin mRNA S₁ probe labelled at an *Eco*RI site at the beginning of the last exon. The 3'-end signal for rabbit β -globin mRNA is approximately 200 nucleotides. The S₁ probe (~20 c.p.m., specific activity 3,000 Ci mmol⁻¹) was annealed to HeLa cell cytoplasmic RNAs (~20 μ g) in 30 μ l of 80% formamide, 0.04 M PIPES pH 6.8, 0.4 M NaCl, 0.1 mM EDTA by denaturation at 80 °C for 10 min, then 53 °C overnight. Ice-cold S₁ buffer (0.3 ml; 0.25 M NaCl, 0.03 M NaOAc pH 4.6, 2 mM ZnSO₄, 50 μ g ml⁻¹ denatured sonicated carrier DNA) plus S₁ (3,000 U) was quickly added to each hybridization and incubated for 1 h at 30 °C. S₁ reactions were ethanol precipitated and fractionated on denaturing, 7 M urea polyacrylamide gels.

To test which of these two explanations is correct, I investigated the effect of placing RNA polymerase II termination signals between the two α genes. Two termination signals have been identified and characterized for RNA polymerase II genes, one for the sea urchin H2A histone gene^{13,14} and the other for the mouse β -globin gene^{15,16}. However, in both cases, it was necessary to include fairly extensive regions of sequence including both the 3' end of the gene as well as 3'-flanking sequence, since neither terminator signal has yet been localized more precisely. Figure 2a demonstrates the various positions in which these terminators were placed with respect to the duplicated α -globin genes. First, the histone terminator was positioned either between ($\alpha T\alpha$) or after ($\alpha\alpha T$) the two α genes. As a control, a deleted form of the histone terminator which is known to have lost its termination activity¹³ was placed between the two α genes ($\alpha\Delta T\alpha$). Second, the mouse β termination sequences were placed in both orientations between the two α genes

($\alpha\tilde{m}\beta\alpha$ and $\alpha\tilde{m}\beta\alpha$).

Figure 2b shows the primer extension data obtained for this second set of $\alpha\alpha$ pSVod constructs. No co-transfection controls are shown for these experiments, since each primer extension experiment is internally controlled by the ratios of the Tag α and α bands. As indicated, $\alpha T\alpha$ caused an approximately threefold increase in the 3' α - as compared with the 5' α -gene signal while $\alpha\alpha T$ caused a similar threefold increase in the 5' α -gene signal. Thus, placing a terminator sequence between or after the duplicated α genes has a similar effect to deleting either of the two α -gene promoters. The control $\alpha\Delta T\alpha$ with a nonfunctional termination signal¹³ gave more equal ratios of 5' and 3' α signals, demonstrating that the 3:1 ratio for 5' α to 3' α in $\alpha T\alpha$ is caused by a termination process. In fact, the 5' α signal is routinely lower than the 3' α signal in $\alpha\alpha$ pSVod, possibly due to the Tag sequence which may reduce the efficiency of the α -gene promoter. Finally, $\alpha\tilde{m}\beta\alpha$ gave threefold more 3' α signal

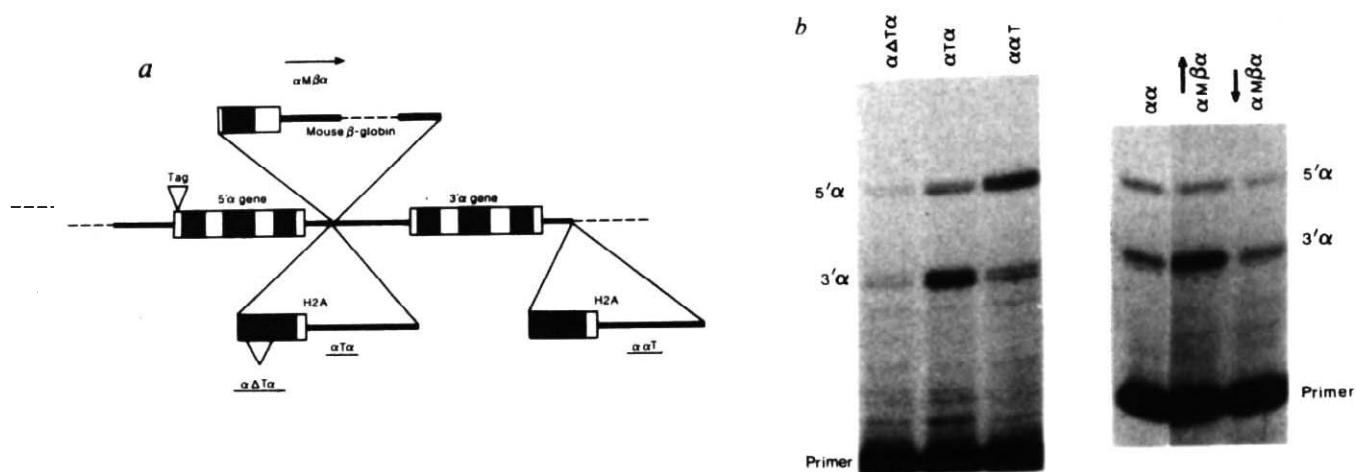


Fig. 2 *a*, Diagram showing positions of H2A histone and β -globin gene termination signals with respect to the two genes in α pSVod. The mouse β -globin terminator was placed in both orientations between the two α genes. The arrow denotes the normal direction of transcription on the β -globin gene. Key as for Fig. 1*a*. *b*, Primer extension RNA analysis on α pSVod terminator transfection as in Fig. 1.

Methods. **DNA constructions:** H2A histone terminator: A 0.9-kb *Taq*I fragment containing the 3' side of the sea urchin H2A histone gene together with nearly all of its 3'-flanking sequence was purified from a larger plasmid containing the whole gene (a generous gift from Professor M. Birnstiel, University of Zurich). This fragment has been shown to contain all of the necessary signals to elicit transcriptional termination (ref. 13). The histone H2A gene *Taq* fragment (T) was then added to either of the *Pst*I sites flanking the 3' α 1-globin gene ($\alpha T\alpha$ - and $\alpha T\alpha$ pSVod). Finally, $\alpha T\alpha$ was cut with *Sac*I and *Pvu*II to excise a region of the histone gene that is essential for transcriptional termination (ref. 13). Mouse β major globin terminator: An *Eco*RI-*Bgl*III DNA fragment containing the whole mouse β -major globin gene plus extensive 3'-flanking sequence was subcloned into pSVed (ref. 24) between the *Eco*RI and *Hinc*II sites (*amp* gene): m β majpSVed. A *Pst*I fragment was then excised from m β majpSVed which extended from the 3' end of intron 2 to the *Pst*I site in pSVed 300 bp beyond the *Hinc*II site. This fragment contains the essential D, E and F fragments defined by Falck-Pedersen *et al.*¹⁶ as well as 300 bp of pBR322 *amp* resistance gene sequence. The 2.0 kb *Pst*I fragment was then ligated into the *Pst*I site between the two α genes in α pSVod in both orientations to form $\alpha \beta \alpha$ - and $\alpha \beta \alpha$ pSVod.

while $\alpha \beta \alpha$ and $\alpha \alpha$ gave nearly equal levels of 5' and 3' α signal. These results confirm the data obtained for $\alpha T\alpha$ by demonstrating that a second terminator sequence, that from the mouse β -globin gene, behaves exactly as the histone terminator and, furthermore, that it is orientation specific. Taken together, these data argue that transcriptional interference does occur between the duplicated α genes, rather than a promoter competition effect. In fact, the data indicate that not only does the first α gene interfere with the second α gene, but also that the second α gene, presumably by transcription all around pSVod, in turn interferes with the first α gene. This would suggest that in $\alpha \alpha$ both α genes are subject to equal transcriptional interference, so that the two α gene signals are nearly equal. However, with $\alpha T\alpha$ the second α gene is released from interference but the first is not, resulting in a 1:3 ratio. Finally, with $\alpha \alpha T$, the first α gene, but not the second, is released from interference, giving a ratio of 3:1.

The present studies on the transcription of duplicated α genes in a transient expression assay demonstrate that a nearly threefold transcriptional interference effect occurs between the two adjacent α -globin genes. The observation that the 3'-positioned α gene inhibits the 5' α gene by transcription all the way around the plasmid suggests that transcriptional interference occurs over a distance of at least 3 kilobases (kb). In fact, this possibility is confirmed by the $\alpha \beta \alpha$ pSVod constructs which effectively separate the two α -globin genes by 3 kb and yet clearly demonstrate a transcriptional interference effect between the two α -globin genes (Fig. 2*b*). Clearly, the study of multiple genes in transient expression plasmids must take into account transcriptional interference as a possible cause of modulation in promoter efficiency. The molecular basis of this interference effect is probably due to RNA polymerase II molecules first transcribing the 5' gene and then reading on into the 3' gene. This effectively obscures the promoter of the 3' gene from independent initiation of transcription by other RNA polymerase II molecules. Transcriptional termination would thus alleviate this interference

effect. The observation that both the H2A and mouse β -globin terminator sequences prevent this interference effect is therefore strong confirmatory evidence that these sequences are transcriptional terminators.

The fact that I observe a threefold interference effect in transient expression may not reflect the true physiological levels of this phenomenon. In transient expression, as many as 10,000 copies of the plasmid are generated by replication. Thus, pSVod

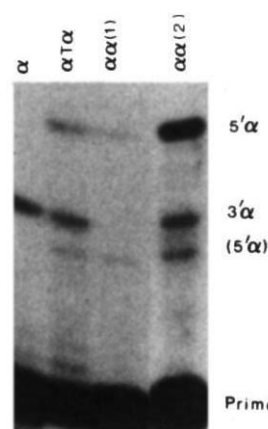


Fig. 3 Primer extension analysis on G418-resistant HeLa cell pools transfected with either α pSVedneo (two separate pools) or $\alpha T\alpha$ pSVedneo. The α lane is a control experiment using RNA purified from the α -globin-producing cell line K562 (ref. 24).

Methods. The $\alpha \alpha$ - and $\alpha T\alpha$ pSVedneo plasmids together with SVpBR328R β 1 as a source of large-T antigen were transfected onto HeLa cells as for Fig. 1. Following exposure to the calcium phosphate-DNA precipitates, the HeLa cells were incubated for 48 h and G418 ($80 \mu\text{g l}^{-1}$) was then added to the medium. After about 2 weeks of G418 selection, small, resistant colonies were obtained and allowed to grow to confluence. Primer extension assays were performed as for Fig. 1, but $\sim 100 \mu\text{g}$ of cytoplasmic RNA was used in each reaction to obtain detectable signals.

possesses an SV40 replication origin that is stimulated by large-T antigen⁵ produced by the R β SVpBR328 co-transfected plasmid. The high copy number of α -globin genes obtained in transfected HeLa cell nuclei may effectively be in excess of the endogenous RNA polymerase II. Transcriptional interference effects would thus be diminished. However, endogenous chromosomal RNA polymerase II genes are usually single copy, so that RNA polymerase II should be in excess. Interference between adjacent genes could therefore be much higher. To test this possibility, I transfected α pSVod and α TpSVod plasmids containing the neomycin drug resistance gene (*neo*)¹⁷ as part of the SV40 early gene transcription unit (Fig. 1a and legend) into HeLa cells. Following conditions for selection with the antibiotic G418, pools of resistant HeLa cell clones that had stably integrated the *neo* gene-containing plasmids were obtained, one pool for α TpSVod and two separate pools for α pSVod. In each case, the complexity of each pool was in excess of 100 independent clones. Therefore, any specific chromosomal position effects on one clone should be averaged out. Similarly, clones in which integration of the plasmid occurred either between or within the two α genes should not influence the average levels of α -globin mRNA expression by the whole pool.

Figure 3 shows the primer extension data for RNA purified from the three G418-resistant HeLa cell pools. High amounts of RNA were used in the reverse transcriptase reactions because the level of α mRNA was lower than in the transient expression experiments. As indicated, both α pSVod pools gave a strong 5' α signal relative to the 3' α signal. Indeed, for $\alpha\alpha(1)$, which overall gave rather low α mRNA signals, no 3' α signal was detected at all, while for $\alpha\alpha(2)$, the 3' α signal was 20-fold lower than the 5' α signal. A third α pSVod pool has recently been analysed and shows 5-fold less 3' α signal than 5' α (data not shown). In contrast, with α Tp both 5' and 3' α signals were nearly equivalent. Note that a band (5') was routinely observed below the 3' α signal. Indeed, this band was more noticeable when using the high amounts of RNA necessary to detect α mRNA signal with the transfected HeLa cell pools. This band clearly derives from the 5' α signal, since no such band is visible with the α control lane but is detectable in the 5' α signal shown for $\alpha\Delta P\alpha$ in Fig. 1b. Presumably, this is due to the different RNA secondary structure of the Tag-containing α mRNA. Taken together, these data demonstrate a 5-to-20-fold level of transcriptional interference between the duplicated α genes when placed in a chromosomal environment. This interference can be wholly alleviated by placing the histone terminator between the two α genes.

The demonstration of a threefold interference effect between duplicated α genes in episomal plasmids and the greater effect in chromosomes, clearly underlines the essential role of transcriptional terminators in eukaryotic gene transcription. Although in these experiments I have intentionally placed the two α genes in close proximity (~700 bp apart), it is probable that interference can occur over greater distances, especially since many gene transcription units are longer than 100 kb^{18,19}.

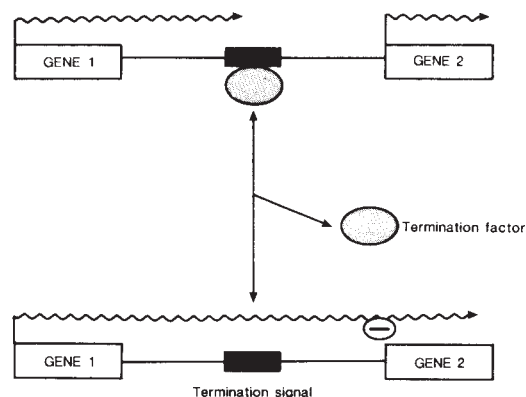


Fig. 4 Model for gene regulation by transcriptional interference.

The interesting possibility that the control of a gene could be partly mediated by transcriptional interference from an upstream gene is now a serious possibility. Figure 4 demonstrates a possible model for this type of control process. The model requires that a termination factor must recognize the termination signal to elicit transcriptional termination. Such an interaction would allow the downstream gene to operate free from upstream gene transcriptional interference. However, the absence or dissociation of the termination factor from the termination signal would allow upstream gene transcripts to read through the terminator to interfere with transcription of the downstream gene. So far, no such terminator factors have been identified for RNA polymerase II genes, although RNA polymerase I terminator factors have recently been identified²⁰. A possible variation on the model depicted in Fig. 4 could be that the factor recognizing the terminator sequence is an anti-termination factor, so that interference of the downstream gene would occur by anti-terminator factor binding rather than terminator factor release. Such terminator and anti-termination factors are well documented in prokaryotes, with the *Escherichia coli* termination factor rho and the bacteriophage λ N and Q antiterminators as classic examples²¹. It remains to be established whether or not equivalent termination factors are involved in the regulation of eukaryotic gene expression.

A number of my colleagues have contributed significantly to this project, Mike Johnson constructed the Tag α gene and did some preliminary experiments²³, Chris Norman constructed the H2A termination constructs, and Sarah Hargreaves carried out primer extension analysis on the α m β α constructs. I also thank the members of my laboratory for many helpful discussions throughout these studies.

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Cloning of a major surface-antigen gene of *Trypanosoma cruzi* and identification of a nonapeptide repeat

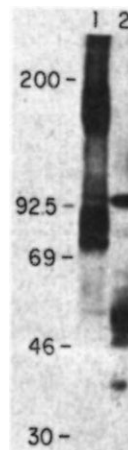
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The parasitic protozoan *Trypanosoma cruzi* can establish infection in humans and other vertebrate hosts through direct penetration of host cells by trypomastigotes transmitted by the insect vector¹. Although the molecular processes involved in trypomastigote interiorization of vertebrate cells are unknown, several studies suggest that surface glycoproteins are involved²⁻⁴. It is likely that the proteins involved are specific to the trypomastigote stage of the parasite, since only trypomastigotes found in both the insect vector and the vertebrate host bloodstream are capable of invading vertebrate cells. In contrast, the epimastigote stage, found exclusively in the vector, and the amastigote stage, an intracellular stage in the vertebrate host, cannot penetrate the cell directly. We have therefore concentrated our efforts on trypomastigote surface proteins and, along with others, have identified two trypomastigote-specific surface glycoproteins of relative molecular mass (M_r) 90,000 (90K) and 85,000 (85K)^{5,6}. Antibody neutralization experiments indicate that the 85K glycoprotein is necessary for efficient interiorization of trypomastigotes in mammalian cells. Here we describe the molecular cloning of a genomic DNA fragment that encodes antigenic determinants present in the 85K trypomastigote surface antigen. The polypeptide fragment encoded by the cloned DNA is recognized by serum from a *T. cruzi*-infected host and is inferred by DNA sequence analysis to contain a nonapeptide unit that is tandemly repeated five times. Also, the messenger complementary to the cloned DNA fragment is present only in the trypomastigote stage of the parasite.

We have previously reported that the major surface proteins of the trypomastigote stage can be isolated free of most cytosolic components by using iminobiotin-avidin interaction⁶. This preparation of iminobiotinylated surface proteins (IBSP) contains

Fig. 1 Immunoblot analysis of SDS-lysates of Peru strain trypomastigotes (lane 1) and Peru strain epimastigotes (lane 2) using anti-IBSP antibodies. Total cellular proteins were solubilized in Laemmli sample buffer¹⁵ at a concentration of 2×10^8 cells ml^{-1} . After being heated for 3 min, 25 μl of each lysate was added to each lane of a 10% polyacrylamide SDS gel. After electrophoresis the gel was electroblotted to nitrocellulose and processed as described previously⁶. The serum used to probe the filter was a 1:1,000 dilution of rabbit anti-IBSP serum. Detection of the rabbit antibody was with ¹²⁵I-goat-anti-rabbit IgG. Markers for calibration of the gel are ¹⁴C-methylated myosin (200,000), phosphorylase-b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000).



the major 85K and 90K glycoproteins as well as several minor surface polypeptides. Sera from rabbits immunized with the IBSP preparation recognized polypeptides of 90K, 85K and 76K in trypomastigotes, but not in epimastigotes, as determined by Western blot analysis (Fig. 1). In contrast, a 105K antigen was recognized in both forms of the parasite.

To obtain DNA fragments which encode a portion of the surface polypeptides, a recombinant DNA library was constructed in the expression vector $\lambda\text{gt}11$ (ref. 7) using fragments of Peru strain trypomastigote genomic DNA (Fig. 2a). Approximately 100,000 recombinant phage were screened with the anti-IBSP sera and 6 phage expressing *T. cruzi* antigens were identified. One of these clones, Tcg-1, encoded a hybrid protein of M_r 132,000, which is approximately 16,000 daltons larger than β -galactosidase. In Western blots the fusion protein was recognized by the anti-IBSP sera as well as by sera from a mouse infected with *T. cruzi*, thus indicating that the epitope(s) encoded in Tcg-1 is also recognized by the immune system of the infected host during an active *T. cruzi* infection (Fig. 2b).

Since the anti-IBSP sera recognized several surface proteins, it was necessary to determine which surface protein was represented in the Tcg-1 fusion protein. BALB/c mice were immu-

Fig. 2 a, Construction and screening of a trypomastigote genomic DNA library. DNA of high M_r was isolated from culture-form trypomastigotes of the Peru strain, partially digested with endonuclease *Hae*III, size fractionated by centrifugation on a 10–40% sucrose gradient and fragments with an average length of 2,000 bp were collected. After a 30 min digestion with slow *Bal*31 nuclease, 5 μg DNA per unit of enzyme (International Biotechnologies Inc.), the resected DNA fragments were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I, and *Eco*RI linkers were added to the ends of the fragments to allow insertion into the single *Eco*RI site of $\lambda\text{gt}11$ ⁷. The recombinant molecules were packaged *in vitro* and plated on *E. coli* strain Y1090. Using 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside as an indicator, recombinant phage represented 85% of all plaques. Approximately 100,000 plaques were screened for antigen producers using a 1:500 dilution of rabbit anti-IBSP sera as described previously¹⁶ except that ¹²⁵I-protein A was used to detect λ plaques recognized by the anti-IBSP sera. Six plaques produced positive signals upon successive rescreens with the anti-IBSP sera. b, Analysis and partial purification of the fused polypeptides encoded in Tcg-1. Tcg-1 hybrid protein and native β -galactosidase were partially purified as described by Hall *et al.*¹⁸. The proteins isolated from the induced lysogens were electrophoresed on a 10% polyacrylamide SDS gel at a concentration of 15 μg protein per lane. After electrophoresis, lane 1 was stained with Coomassie blue and lanes 2–5 were electroblotted to nitrocellulose. In lane 1 the arrow denotes the position of the fusion protein at 132,000 M_r . Lanes 2 and 3 are Western blots of Tcg-1 fusion protein reacted with anti-IBSP sera (lane 2) and sera from a mouse infected with *T. cruzi* (lane 3). Partially purified β -galactosidase from $\lambda\text{gt}11$ was Western blotted and reacted with anti-IBSP sera (lane 4) and *T. cruzi*-infected mouse sera (lane 5).

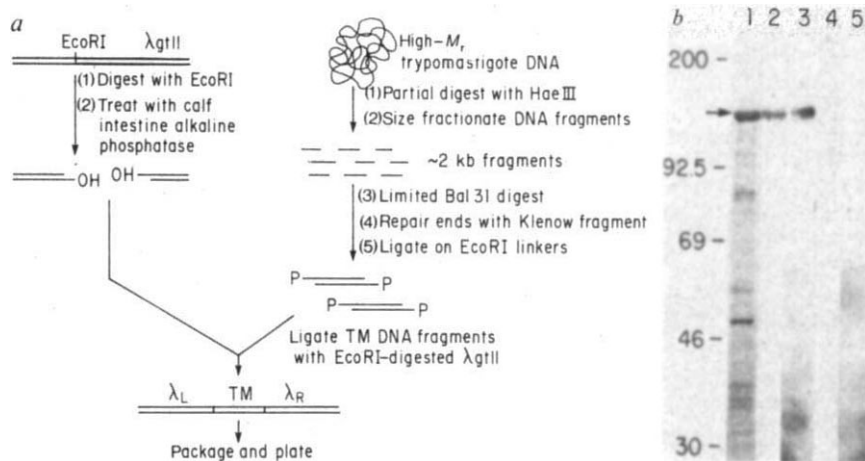
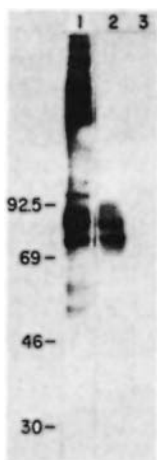


Fig. 3 Identification of native proteins that share antigenic determinants with Tcg-1 fusion protein. Western blots of a trypanomastigote lysate (lane 2) and epimastigote lysate (lane 3). Lane 1 shows a Western blot of a trypanomastigote lysate probed with anti-IBSP sera at a 1:1000 dilution. **Methods.** Tcg-1 fusion protein (270 μ g) was electrophoresed on a preparative 10% polyacrylamide SDS gel and electroblotted to nitrocellulose. Two strips of the extreme ends of the nitrocellulose blot were removed and probed with anti-IBSP sera to determine the precise location of the Tcg-1 fusion protein. The region of the nitrocellulose containing the fusion protein was excised and reacted with 5 ml of undiluted anti-IBSP sera plus 5 ml of blocking buffer as described previously⁵. After 10 washes in buffer containing 10 mM Tris pH 7.4, 0.9% saline, 0.05% Tween-20 and 0.01% gelatin, the bound antibody was eluted by incubation in 1.0 ml of 0.1 M glycine-HCl pH 2.6, 0.15 M NaCl and immediately neutralized by addition of an equal volume of 1 M Tris-HCl pH 8.0. This antibody solution was used to probe the Western blots.



nized with a protein fraction enriched for Tcg-1 fusion protein. The mouse anti-Tcg-1 sera were absorbed against induced λ gt11 lysogens and used to probe lysates of trypanomastigotes and epimastigotes on Western blots (not shown). While no polypeptides in the epimastigote lysate were recognized by the serum antibodies, two polypeptides of M_r 85,000 and 76,000 were recognized in the trypanomastigote lysate. However, the intensities of the bands in the autoradiogram were weak, suggesting that a strong immune response was not elicited against the fusion protein. We used antibody selection⁸ to confirm the identity of the *T. cruzi* polypeptide encoded in Tcg-1. Tcg-1 fusion protein bound to nitrocellulose was used to affinity-purify antibodies from rabbit anti-IBSP sera. The affinity-purified antibodies recognized two polypeptides of 85K and 76K in Western blots of a trypanomastigote lysate (Fig. 3). However, no polypeptides were detected in Western blots of epimastigote lysates. In a control experiment using native β -galactosidase under the same conditions as for antibody selection with Tcg-1 fusion protein, no polypeptides in either of the *T. cruzi* lysates were recognized. We conclude from these results that Tcg-1 contains DNA sequences that encode an epitope(s) present in the 85K and 76K polypeptides.

As shown in Fig. 1, both the 85K and 76K polypeptides are

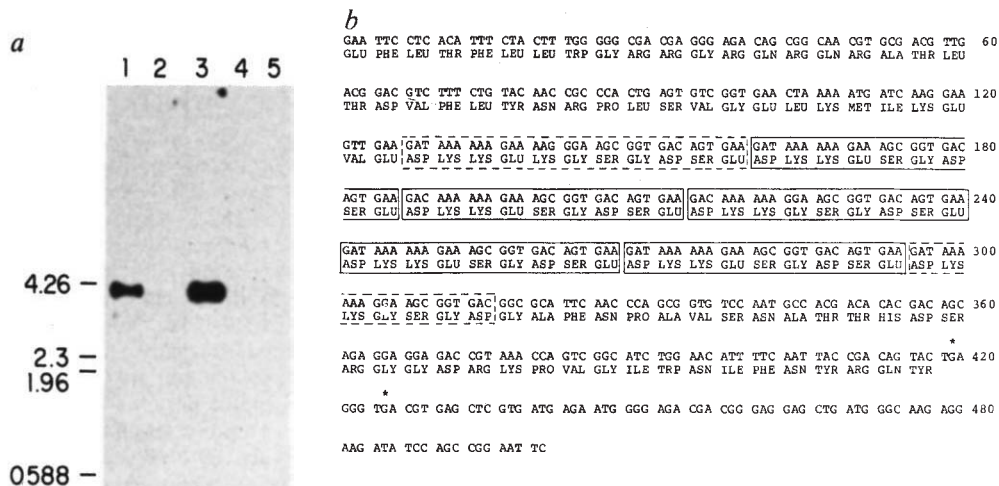
present in the trypanomastigote but not epimastigote stage of the parasite. To determine whether the mRNA(s) which code for these polypeptides are also stage specific, the 500-base-pair (bp) *T. cruzi* DNA fragment in Tcg-1 was hybridized to a Northern blot containing poly(A)⁺ RNA and/or total cytoplasmic RNA extracted from trypanomastigotes, epimastigotes and amastigotes. Hybridization was observed to a single mRNA band with an apparent molecular length of 3.8 kilobases (kb) only in trypanomastigote RNA, thus confirming the stage specificity of these polypeptides (Fig. 4a).

The *T. cruzi* fragment in Tcg-1 was excised by cleavage with endonuclease *Eco*RI, re-cloned into the sequencing vector M13mp9 and sequenced by the Sanger dideoxy chain-termination method⁹. The DNA fragment encoding the surface antigen was 500 bp long, and contained a 27-bp repeat unit which occurred five times in tandem within the sequence (Fig. 4b). Degenerate repeats of 33 bp and 21 bp preceded and followed the five tandem repeats, respectively. Within the repeat unit, a single base change of A $\rightarrow$ G occurred once (nucleotide 223) and a T $\rightarrow$ C transition twice (nucleotides 189 and 215).

Since no protein sequence data are available for *T. cruzi* surface proteins, the amino-acid sequence encoded in the *T. cruzi* DNA fragment in Tcg-1 was deduced from the DNA sequence. The coding strand was determined by hybridization of poly(A)⁺ trypanomastigote RNA to M13mp9 recombinants containing the two complementary DNA strands. A single open reading frame encoding 139 amino acids in frame with β -galactosidase was observed. The predicted reading frame terminated with a TGA stop codon immediately followed by a second TGA codon 3 bp downstream. In this reading frame the A $\rightarrow$ G transition occurred in the second base of the code, resulting in a Glu $\rightarrow$ Gly amino-acid change. The T $\rightarrow$ C transition resulted in a third position change in Asp, but the amino acids remained unchanged. With regard to the two degenerate repeat units, the 33-bp unit was generated by the insertion of an additional Lys-Gly between amino acids 4 and 5 while the 21 bp partial repeat terminated prematurely at amino acid 7.

In summary, clone Tcg-1 encodes antigenic determinants present in the trypanomastigote 85K surface antigen as well as a 76K protein. The relationship between the 85K and 76K proteins is unclear, but it is possible that the 76K protein represents either a precursor or breakdown product of the 85K surface protein. The observation that a 76K protein is not present in the IBSP preparation used in these studies and that the Tcg-1 DNA insert hybridizes to a single- M_r RNA argues in favour of one rather than two polypeptides being represented by the *T. cruzi* DNA insert present in Tcg-1.

Fig. 4 a, Northern blot of poly(A)⁺ RNA from trypanomastigotes (lane 1) and epimastigotes (lane 2), and total RNA from trypanomastigotes (lane 3), epimastigotes (lane 4) and amastigotes (lane 5) hybridized to the 500-bp Tcg-1 insert DNA nick-translated with [α -³²P]dCTP to a specific activity of 2×10^8 c.p.m. per μ g. Processing of the blot was as described previously¹⁷. **b**, Nucleotide sequence of the genomic DNA insert in Tcg-1. The sequence was determined by use of the dideoxy chain-termination method⁹ after subcloning the *Eco*RI fragment of Tcg-1 into M13mp9 in both directions¹⁸. The reading frame is shown in phase with β -galactosidase and the TGA termination codons are denoted by an asterisk (*). The repeat unit is indicated by the boxed regions and the partial repeat units are included in broken boxes. Coding strand preference was determined by hybridization of recombinant M13mp9 DNA with poly(A)⁺ trypanomastigote RNA (data not shown).



One of the most interesting features of the Tcg-1 antigen is the presence of the tandem repeating regions; these are strikingly similar in architecture to tandem repeating regions in both the S-antigen and the circumsporozoite surface (CS) protein of *Plasmodium*¹⁰⁻¹⁴. As in the malarial proteins, the repeat unit is well conserved, with some base changes occurring in the third base of the codon, and thus no concomitant change occurring in the amino acid. Also, the repeat unit is rather small, with the more homogeneous core repeats being flanked by degenerate repeats. In addition, the repeat units are found in surface or secreted proteins whose synthesis is stage specific.

It would be of particular interest to determine the degree to which the repeat unit is conserved among different *T. cruzi* strains. It is known that the repeat units present in the CS protein of two strains of *Plasmodium knowlesi*¹² and the repeat units present in the S-antigen of two strains of *Plasmodium falciparum*¹⁴ are totally divergent and share essentially no sequence homology. If this should be the case for the repeat unit in the 85K trypomastigote surface protein, the repeat unit might serve as a convenient means of identification of specific strains of the parasite by direct immunodiagnostic techniques.

We do not know what role the repeat unit may have in the biological function of the 85K surface protein, nor the function of the 85K protein in the life-cycle of the parasite. However, a

more thorough knowledge of the conservation of the repeat among different *T. cruzi* isolates and the immunological properties of the repeat should elucidate its usefulness to the survival of the parasite in the host as well as its potential as a diagnostic antigen and/or a vaccine.

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Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*

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The Rhizobiaceae family of bacteria is characterized by the ability to form cortical hypertrophies on plants, and the ability to re-isolate the bacteria from these galls or nodules¹. This family includes the genera *Rhizobium*, *Bradyrhizobium*, *Agrobacterium* and *Phyllobacterium*. Another unique feature of these bacteria is that they contain two forms of the enzyme glutamine synthetase, termed GSI and GSII^{2,3}. GSI is typical of prokaryotic glutamine synthetases with respect to enzyme structure, the modulation of activity by post-translational modification, immunological cross-reactivity, and amino-acid sequence^{2,4,5}. By contrast, GSII is distinct from all other known prokaryotic glutamine synthetases in structure and immunological reactivity, and is not known to be post-translationally modified. In these respects GSII is similar to eukaryotic glutamine synthetases⁶. We have isolated and characterized the gene encoding GSII, which we term *glnII*, from *Bradyrhizobium japonicum*, the soybean symbiont. We show here that the amino-acid sequence of GSII, as inferred from the gene sequence, is highly homologous to plant glutamine synthetases, suggesting that this bacterial gene is of eukaryotic origin.

The GSII enzyme was purified as described in the Table 1 legend. This preparation was >95% pure as indicated by SDS-polyacrylamide gel electrophoresis. The amino-acid sequence of the amino terminus of GSII was determined by sequential Edman degradation and was used to design a mixed oligonucleotide probe with homology to the DNA which encodes the first six amino acids of GSII (Fig. 1a). An ambiguity at position 16 of the oligonucleotide was not included because two of the leucine codons, UUA and UUG, are infrequently utilized in *B. japonicum*. Two cosmids, pRJcos7-20 and pRJcos13-79, were isolated from a *B. japonicum* library constructed in the cloning vector pLAFRI^{7,8} by hybridization with radiolabelled oligonucleotide^{9,10}. The region of hybridization in pRJcos7-20 was

Table 1 Purification of GSII

Fraction	GSII activity (units)	Total protein (mg)	Specific activity (units mg ⁻¹)	Purification factor	Yield (%)
Extract	149	48	3.10	(1)	(100)
Affi-Gel Blue	39.1	0.89	43.9	14	26
DEAE HPLC fraction 17	9.92	0.12	82.7	27	6.7

Bradyrhizobium japonicum USDA 110 was grown to mid-log phase in 2 litre of MBX medium⁵ with 10 mM glutamate. Cell extracts were prepared by the method of Tronick *et al.*⁴ using GS buffer (GSB is 10 mM imidazole-HCl pH 7.15 and 1.0 mM MnCl₂). GS activity is expressed in units of μmol of γ -glutamylhydroxamate per min. as measured by the reverse transferase assay²³. Heat inactivation of GSII was carried out on undiluted extracts by incubation at 58 °C for 30 min. In the extract used for purification, GSII accounted for 88% of the total GS activity. All purification steps were done at 4 °C. The extract was diluted with GSB to a protein concentration of 1 mg ml⁻¹ and loaded on a 10 ml Affi-Gel Blue (Bio-Rad) column at a flow rate of 0.5 ml min⁻¹. The column was washed with 50 ml of GSB and eluted with a 50-ml linear gradient of 0-5 mM ATP in GSB. A broad peak of GS activity, including both GSI and GSII, eluted immediately upon the initiation of the ATP gradient. The fractions with the highest specific GS activity were combined and loaded onto a Bio-Rad Bio-Gel TSK DEAE-5-PW ion-exchange HPLC column (75 × 7.5 mm) equilibrated with GSB, at a flow rate of 1 ml min⁻¹. The column was eluted with a 40-ml linear gradient of 0-500 mM KCl in GSB. The first and second peaks of GS activity corresponded to GSII and GSI respectively. Fractions (1 ml) for sequence determination were desalted on a 10 ml Bio-Rad P-6 column and lyophilized.

localized to 5.0 kilobase-pair (kbp) *Eco*RI, 1.0 kbp *Bgl*II and 2.1 kbp *Sal*I fragments. These fragment sizes agree with those detected by hybridization to Southern transfers¹¹ of total genomic DNA restriction digests.

The 2.1-kbp *Sal*I fragment with homology to the oligonucleotide probe was subcloned in both orientations in pBR322¹². The resulting plasmids, pBJ196A and pBJ196B, were tested for their ability to complement glutamine auxotrophy in *Escherichia coli* ET8051 [$\Delta(rha-glnA) hutC_K rbs nal^+$]¹³. ET8051/pBJ196A grew well on defined medium plates with ammonia as the sole nitrogen source and yielded extracts with 0.82 units of GS activity per mg of protein. This GS activity was completely heat labile, a property specific to GSII, and co-sedimented with the GSII activity of *B. japonicum* in sucrose density gradient centrifugation. These data indicate that the entire *glnII* gene is located on the 2.1-kbp *Sal*I fragment insert of pBJ196A. ET8051/pBJ196B, ET8051/pRJcos7-20 and the control strain ET8051/pBR322 gave no detectable growth on defined-medium

Fig. 1 a, The sequence of the amino terminus of GSII and the sequence of the mixed oligonucleotide hybridization probe used for gene identification. **b**, The complete DNA sequence of the coding region of *glnII* and the predicted amino-acid sequence of GSII.

Methods. Protein sequencing was performed on an Applied Biosystems Model 470A gas phase sequencer at the University of Michigan protein sequencing facility. Oligonucleotide probes were synthesized by the phosphoramidite method on an Applied Biosystems Model 380A synthesizer. Oligonucleotides were purified by denaturing 12% polyacrylamide gel electrophoresis²⁴. DNA was sequenced by the dideoxy-nucleotide chain termination method²⁵. Random fragments were generated by sonication²⁶ and subcloned into the *Sma*I site of M13-mp19²⁷. All regions were sequenced either on both strands or from three different fragments of the same strand. Plasmids were tested for their ability to complement glutamine auxotrophy in *E. coli* ET8051 on M9 defined-medium agar plates²⁸ with 0.2% glucose and 1 mM thiamine. GS1 and GSII were separated by sucrose density gradient centrifugation as described previously⁵ except that the centrifugation was carried out at 50,000 r.p.m. for 3 h at 4°C.

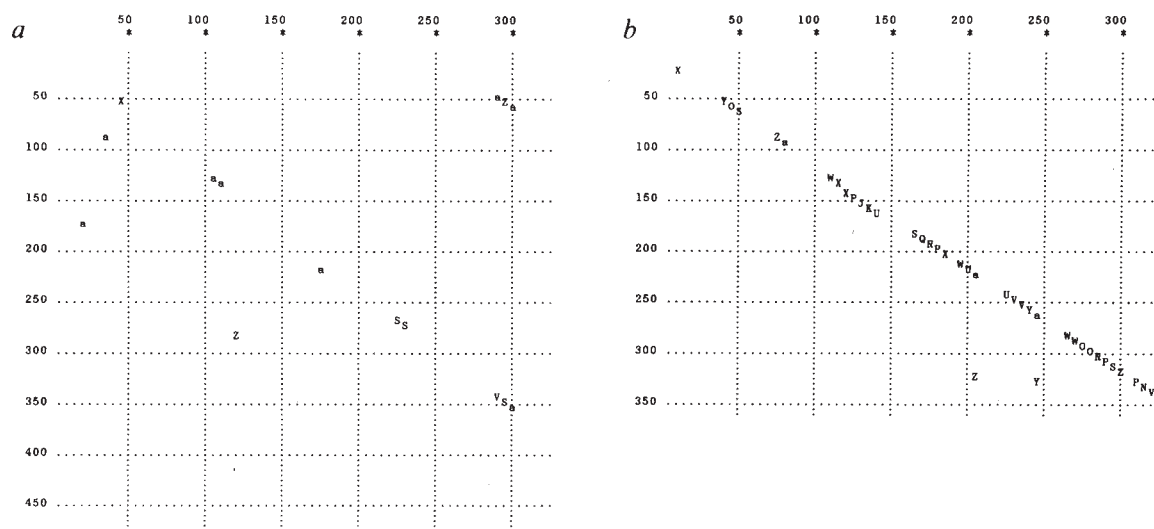
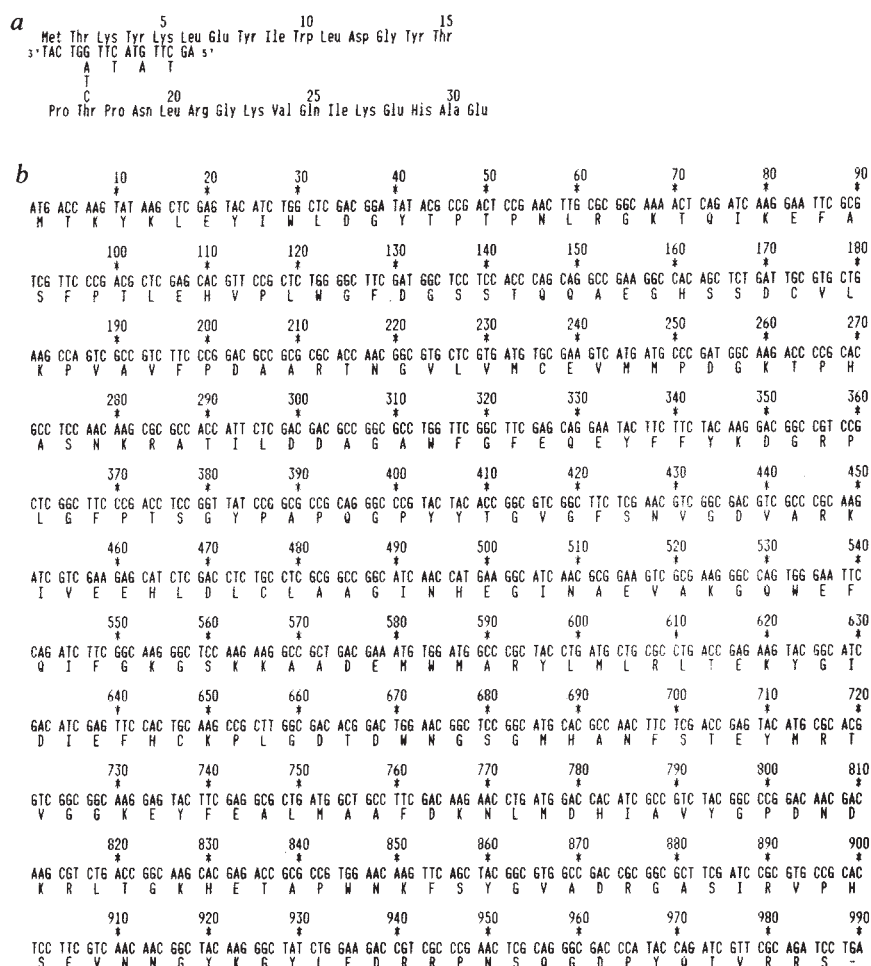


Fig. 2 Amino-acid homology matrices comparing *B. japonicum* GSII (x axis) to *Anabaena* 7120 (y axis, panel a) and *Phaseolus vulgaris* root GS (y axis, panel b). Homology matrices were plotted with the Pustel and Kafatos sequence analysis program¹⁷ using the following parameters: range = 11, scale factor = 0.75, minimum value = 47, compression = 5. Each letter represents a 2% extent of homology in that region of the matrix (A = 100–99%, B = 98–97% ... Z = 50–49%; a = 48–47%).

plates and produced no detectable glutamine synthetase activity. This indicates that the complementation observed with ET8051/pBJ196A was due to *glnII* expression from the *tet* promoter of the vector, in agreement with the gene orientation as indicated by sequence determination (see below).

In order to determine the precise location of the *glnII* gene, we sequenced the 2.1-kbp *Sal*I fragment carried in pBJ196A.

The DNA sequence contains a single long open reading frame which encodes GSII (Fig. 1b). The relative molecular mass of the subunit of GSII, predicted by the complete amino-acid sequence, is 36,904, in good agreement with 36,000 as determined by SDS-polyacrylamide gel electrophoresis¹⁴. The amino terminus of GSII matches the protein sequencing data in 28 of 31 positions. The discrepancies at positions 24, 29 and 31 are

Table 2 Statistical significance of amino-acid sequence homologies between *B. japonicum* GSII and other glutamine synthetases

Glutamine synthetase source	% Identity to GSII	Similarity score	z value
<i>Bradyrhizobium japonicum</i> GSII	100	1719	208.4
<i>Phaseolus vulgaris</i> ¹⁶	43.8	663	77.3
<i>Pisum sativum</i> *	46.6	642	73.9
<i>Medicago sativa</i> ²¹	43.8	674	78.9
<i>Nicotiana plumbaginifolia</i> *	42.6	667	74.0
<i>Anabaena</i> 7120 ¹³	24.4	114	8.83

All scores are from optimized alignments calculated with ktup = 1 and 1,000 randomized sequences using the RDF program of Lipman and Pearson¹⁸. The z value equals the similarity score minus the mean of the similarity scores with randomized sequences divided by the standard deviation of the randomized comparisons. A similarity score is considered significant if its z value is greater than 10 while z values less than 3 are not significant.

* G. Coruzzi, S. Tingey and B. Walker, personal communication.

presumably due to inaccuracies in the final cycles of the protein sequencing.

The comparisons of the *B. japonicum* GSII amino-acid sequence with the *Anabaena* 7120 GS¹⁵ and the *Phaseolus vulgaris* root GS¹⁶ sequences are shown as homology matrixes in Fig. 3. These matrixes were generated using the analysis program of Pustell and Kafatos¹⁷ with parameters set so that each letter within the matrix represents a match of 47%, or greater, over a span of 23 amino acids. It can be seen that GSII has only limited homology to the bacterial GS (Fig. 3a) but extensive homology to the plant GS (Fig. 3b).

GSII was compared with a variety of glutamine synthetases using the method of Lipman and Pearson¹⁸ which optimizes the alignment between amino-acid sequences and quantitates the significance of the similarity (Table 2). Despite extensive homology among most prokaryotic glutamine synthetases, the homology between GSII and the typical bacterial glutamine synthetase of *Anabaena* 7120 is only marginally significant. In contrast, GSII has extensive homology with all eukaryotic glutamine synthetases that we have examined. Because all suggested cases of convergent evolution result in similarities of enzyme function without extensive sequence homology¹⁹, and because GSII is found only in the Rhizobiaceae³, we conclude that the *B. japonicum* *glnII* gene is the result of a eukaryote to prokaryote gene transfer event. We suggest that a plant served as the source of the progenitor *glnII* gene because of the plant pathogenic nature of the Rhizobiaceae.

The presence of the *glnII* gene in the Rhizobiaceae is the first evidence of gene transfer to symbiotic bacteria from the eukaryotic host. Another suggested example of eukaryote to prokaryote gene transfer, involving bacteriocuprein of *Photobacterium leiognathi*¹⁹, has recently come under dispute²⁰.

Although sequence homology suggests the origin of the *glnII* gene, questions pertaining to the mechanism, frequency or function of gene transfer between symbionts or pathogens cannot be directly addressed. Since plant GS genes are known to contain introns²¹, *B. japonicum* *glnII* had to evolve further by the loss of the introns, or the gene transfer must have occurred prior to the acquisition of introns in the plant genes. In addition, there is no obvious reason why the acquisition of a eukaryotic gene would confer a selective advantage on a plant symbiotic bacterium. GSII is not known to serve an essential function, acting only to provide extra ammonia assimilatory capacity during growth under nitrogen limited conditions²². Nevertheless, it appears that the ability to acquire genetic information from eukaryotes can be a source of genetic diversity in the evolution of bacteria.

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Ultrafast carotenoid to pheophorbide energy transfer in a biomimetic model for antenna function in photosynthesis

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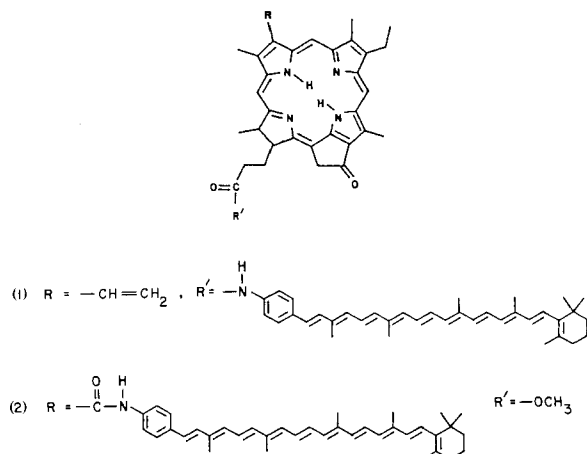
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Carotenoids serve as light-harvesting pigments and as photoprotective agents in photosynthetic organisms¹⁻⁹. Their role as antenna pigments involves absorption of photons in the blue-green spectral region followed by highly efficient singlet-singlet energy transfer to a neighbouring chlorophyll. The dependence of both the rate and mechanism of energy transfer on carotenoid-chlorophyll distance and orientation is unknown. Here, we have directly measured both the rate and efficiency of singlet energy transfer from a carotenoid covalently linked to pyropheophorbide *a* (PPheo *a*) in two model compounds, using picosecond transient absorption spectroscopy. In one model the π systems of the carotenoid and PPheo *a* possess a maximum edge-to-edge distance of 5 Å, while in the other model this distance is only 2 Å. Energy transfer occurs from the carotenoid to PPheo *a* at the 2-Å distance with a rate constant of $7 \pm 2 \times 10^{10} \text{ s}^{-1}$ and $53 \pm 5\%$ efficiency, while energy transfer at the 5-Å distance occurs at a rate constant of $< 3 \times 10^9 \text{ s}^{-1}$ and with $< 5\%$ efficiency. These results provide evidence that short distances and strong electronic interactions between carotenoids and chlorophylls are necessary to achieve the high energy transfer efficiencies observed *in vivo*.

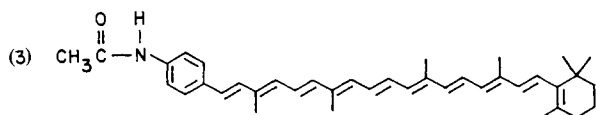
The lifetimes of the lowest excited singlet state, S_1 of several carotenoids including β -carotene have been measured recently by transient absorption spectroscopy after direct excitation of the carotenoids with 4-ps pulses of 510-nm light¹⁰. That study showed that a combination of an unusually small radiative rate with an extremely fast rate of internal conversion results in carotenoid lifetimes that are of the order of 10 ps (for example, 8.4 ps for β -carotene). The short lifetimes of these states suggest that the rate of energy transfer from carotenoids to chlorophylls required to achieve very efficient energy transfer must be $>10^{11} \text{ s}^{-1}$. This requirement further implies that the electronic interaction between the carotenoid donor and the chlorophyll acceptor must be very strong¹¹⁻¹⁴.

The two model compounds that we studied (see below) differ in that the carotenoid moiety of compound (1) is linked to the



7-propionic acid side chain of the PPheo *a* via an amide bond, whereas in compound (2) the 2-vinyl group of methyl pyropheophorbide *a* has been replaced by a carboxylic acid which in turn has been linked to the same 4-aminophenyl carotenoid via an amide linkage. High-resolution ^1H -NMR spectroscopy of compounds (1) and (2) establishes that the time-averaged orientation of the carotenoid relative to the PPheo *a* in both (1) and (2) is such that the carotenoid is extended away from the macrocycle, rather than tightly folded back across the PPheo *a*. Thus, the maximum edge-to-edge distance between the carotenoid and PPheo *a* π system in (1) is $\sim 5 \text{ \AA}$, while that in (2) is $\sim 2 \text{ \AA}$. Moreover, in compound (2) the amide bond possesses a resonance structure which allows a degree of direct conjugation between the carotenoid and PPheo *a* π systems.

The rate and efficiency of energy transfer from the carotenoid to PPheo *a* in compounds (1) and (2) were determined directly by picosecond transient absorption spectroscopy. For this, solutions of compounds (1), (2) and carotenoid acetamide (3) (see below) were prepared in deoxygenated, spectral-grade toluene



and placed in cells with a 2-mm pathlength ($A = 0.8$). The optical absorption spectra of compounds (1) and (2) can be closely approximated as a simple superposition of the spectrum of compound (3) with that of the respective PPheo *a* derivative.

Figure 1 shows the transient absorption spectra of compounds (1)–(3) obtained at 0 ps relative to a 4-ps, 515-nm laser flash. In each case the carotenoid band is strongly bleached with a positive absorbance appearing near 560 nm. Similar spectral features have been observed previously for β -carotene and related carotenoids and are characteristic of carotenoid S_1 formation¹⁰.

Figure 2 shows the behaviour with time of the carotenoid

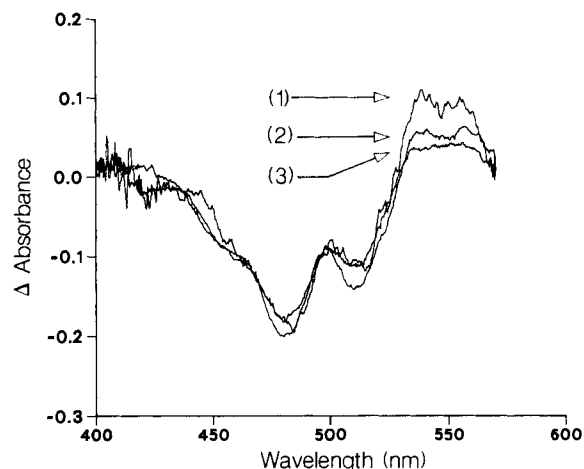


Fig. 1 Transient absorption spectra of the indicated compounds at 0 ps relative to a 4-ps, 25- μJ , 515-nm laser flash.

Methods. The 610-nm, 0.4-ps, 0.5-nJ output of a mode-locked Ar^+ -synchronously pumped R6G/DQOCI dye laser was amplified to 1.5 mJ using a four-stage R640 dye amplifier pumped by a frequency-doubled Nd-YAG laser operating at 10 Hz. The amplified laser pulse was split with a dichroic beam splitter. A 610-nm, 0.5-ps, 0.8-mJ pulse was focused into ethanol to generate a 5- μJ anti-Stokes Raman-shifted pulse at 515 nm. This pulse was amplified to 150 μJ using a two-stage coumarin-500 dye amplifier pumped by a frequency-tripled Nd-YAG laser. During amplification the 515-nm pulse broadened to 4 ps. The remaining 610-nm, 0.5-ps, 0.7-mJ pulse was used to generate a 0.5-ps white-light continuum probe pulse. A 25- μJ , 515-nm pulse was used to excite a spot of 1 mm diameter on the sample cuvette. Absorbance measurements were made in a double-beam configuration which used optical multichannel detection. Delays between pump and probe pulses were achieved using an optical delay line. The absorbance of the carotenoid at 515 nm is ~ 7 times that of PPheo *a* in (1) and (2), and the carotenoid is therefore preferentially excited.

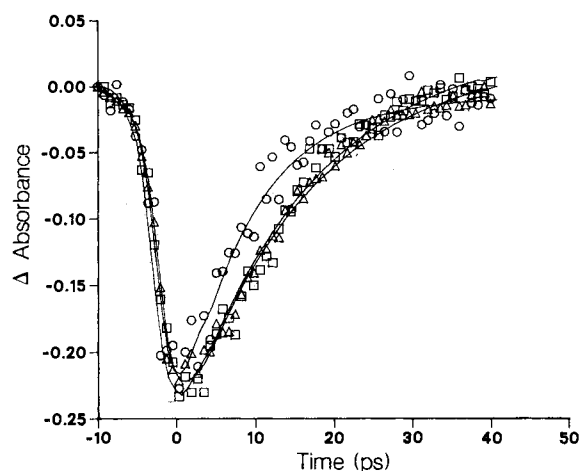


Fig. 2 Decay of the transient absorption at 480 nm of compounds (1) ($\square$), (2) ($\circ$) and (3) ($\triangle$) following a 4-ps, 25- μJ , 515-nm laser flash. The solid curves are the best single exponential fits to the data.

absorbance changes at 480 nm. Each absorbance decays to zero with single exponential kinetics¹⁵, with $\tau = 15.9 \pm 0.8$, 7.6 ± 0.8 and 16.2 ± 0.8 -ps for compounds (1)–(3), respectively. Note that the time constant for recovery of the 480-nm bleach of the carotenoid in (1) is very similar to that of the carotenoid (3) alone, whereas the corresponding time constant for (2) is smaller than that of (3) by more than a factor of 2. From these results it is clear that the carotenoid S_1 population of (2) is depleted by an additional decay pathway that competes effectively with the intrinsic decay of the carotenoid S_1 state, whereas a similar competitive pathway in (1) is much less efficient.

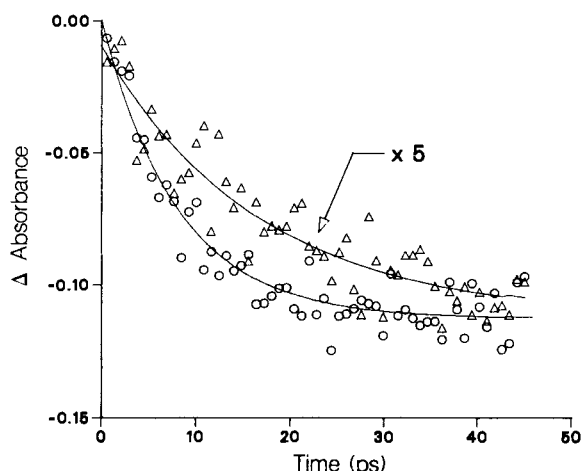


Fig. 3 Appearance of the transient bleach of compound (1) at 670 nm (Δ) and of compound (2) at 675 nm ($\circ$) following a 4-ps, 25- μ J, 515-nm laser flash. The absorbance change data for compound (1) are multiplied by 5 for comparison with those of compound (2). The solid curves are the best single exponential fits to the data.

Because the carotenoid does not absorb light at wavelengths >600 nm, this new decay pathway can be shown to be energy transfer to the attached PPheo *a* by examining the transient absorption changes of the PPheo *a* bands at 670 nm in (1) and at 675 nm in (2) after 515-nm excitation. Figure 3 shows the appearance kinetics of the bleach of these bands. The band at 675 nm in (2) bleaches with a 7.8 ± 0.8 -ps time constant whereas that of (1) bleaches with a 17.2 ± 0.8 -ps time constant. Also, the yield of S_1 of the PPheo *a* in (1) is much smaller than that of (2). The inverse of the time constant for the appearance of the PPheo *a* band bleach, which is the same as that for the recovery of the respective carotenoid bleach, is $k_{et} + k_d$, where k_{et} is the singlet-singlet energy transfer rate constant and k_d is the intrinsic carotenoid S_1 decay rate constant. Rate constant k_d is obtained from the S_1 decay of carotenoid (3). Thus, the rate of energy transfer for (2) is $7 \pm 2 \times 10^{10} \text{ s}^{-1}$, while that for (1) is $<3 \times 10^9 \text{ s}^{-1}$ based on the error limits of the measurements.

These results show that the carotenoid in compound (1) does not transfer energy efficiently to the PPheo *a* bonded to it. Better than 95% of the excited carotenoid molecules decay directly to ground state through their dominant radiationless pathways. On the other hand, the carotenoid in compound (2) transfers energy efficiently to the PPheo *a*. As no other decay mechanisms for S_1 are observed, the energy transfer efficiency is $k_{et}/(k_{et} + k_d) = 53 \pm 5\%$ for compound (2).

In conclusion, our results suggest that very strong electronic coupling between the π systems of a carotenoid donor and a chlorophyll derivative acceptor is necessary to achieve efficient singlet energy transfer rates. Strong electronic coupling between an energy donor and acceptor implies that terms involving orbital overlap must be included in the quantum mechanical description of the energy transfer mechanism¹¹⁻¹⁴. This in turn implies that in natural photosynthetic membranes where the coupling interaction is governed by the three-dimensional structure of the pigment-bearing proteins, the distances between donor and acceptor must approach van der Waals' contact. The successful characterization of singlet energy transfer in a well-defined model system augurs well for future direct measurement of energy transfer kinetics in natural photosynthetic antenna preparations.

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Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA

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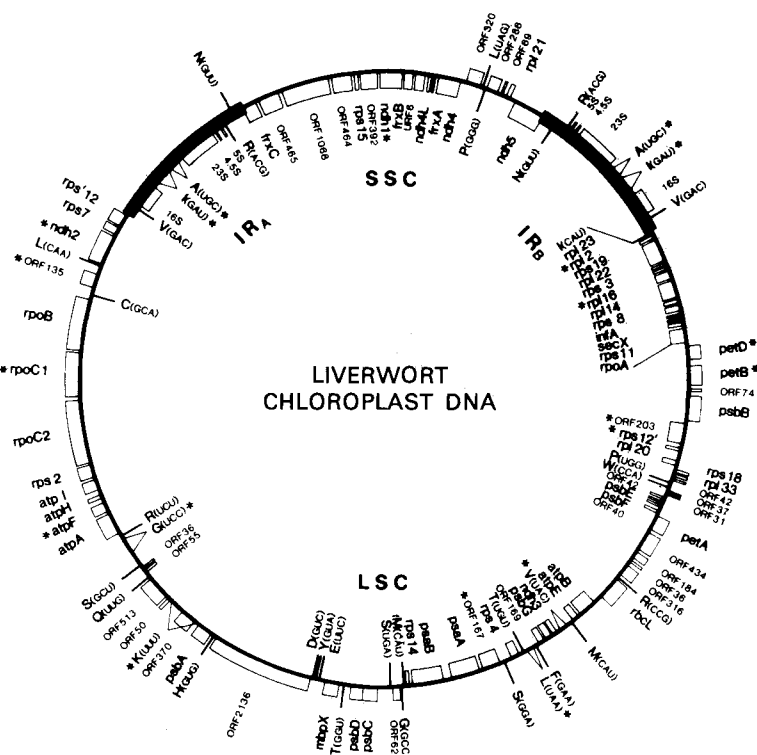
Chloroplasts contain their own autonomously replicating DNA genome. The majority of proteins present in the chloroplasts are encoded by nuclear DNA, but the rest are encoded by chloroplast DNA and synthesized by the chloroplast transcription-translation machinery¹⁻⁴. Although the nucleotide sequences of many chloroplast genes from various plant species have been determined, the entire gene organization of the chloroplast genome has not yet been elucidated for any species of plants. To improve our understanding of the chloroplast gene system, we have determined the complete sequence of the chloroplast DNA from a liverwort, *Marchantia polymorpha*, and deduced the gene organization. As reported here the liverwort chloroplast DNA contains 121,024 base pairs (bp), consisting of a set of large inverted repeats (IR_A and IR_B, each of 10,058 bp) separated by a small single-copy region (SSC, 19,813 bp) and a large single-copy region (LSC, 81,095 bp). We detected 128 possible genes throughout the liverwort chloroplast genome, including coding sequences for four kinds of ribosomal RNAs, 32 species of transfer RNAs and 55 identified open reading frames (ORFs) for proteins, which are separated by short A+T-rich spacers (Fig. 1). Twenty genes (8 encoding tRNAs, 12 encoding proteins) contain introns in their coding sequences. These introns can be classified as belonging to either group I or group II, as described for mitochondria⁵. Interestingly, seven of the identified ORFs show high homology to unidentified reading frames (URFs) found in human mitochondria^{6,7}.

The genes encoding the chloroplast rRNAs (16S, 23S, 4.5S and 5S rRNAs) were localized by Southern hybridization with specific rRNA probes⁸⁻¹⁰. DNA sequences also confirmed the presence of the rRNA operons in both inverted repeat regions. Genes encoding tRNAs were localized using the T ψ loop sequence (GTTTCA) and identified by constructing the familiar

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Fig. 1 Gene organization of the chloroplast genome from a liverwort, *Marchantia polymorpha*. Thick lines indicate the inverted repeats (IR_A and IR_B). SSC and LSC indicate the small single-copy region and large single-copy region, respectively. Genes shown outside the map are transcribed anticlockwise, and those inside are transcribed clockwise. The tRNA genes are identified by the one-letter amino-acid code with their anticodons given in parentheses. Asterisks indicate genes having introns in their sequences. The genes for the 50S and 30S ribosomal proteins are shown as *rpl* and *rps*, respectively. *atp*, *psa*, *psb* and *pet* represent the genes for subunits of H⁺-ATPase, proteins in photosystem I, photosystem II, and cytochrome *b₆/f* complex, respectively. We tentatively designated chloroplast ORFs which exhibited homology to those found in human mitochondria, as *ndh*. ORFs which showed homology to 4Fe-4S proteins as *frx*, and inner membrane permease protein found in *E. coli* as *mbp*. *infA* and *secX* represent genes for initiation factor 1 and functionally unidentified protein expressed in *E. coli*, respectively. *rbcL*, the gene for the large subunit of ribulose biphosphate carboxylase. *rpoA*, *rpoB*, *rpoC1* and *rpoC2* are the genes for RNA polymerase subunits. Ribosomal RNA operons are located in both IR regions (16S, 23S, 4.5S and 5S).

Methods. For DNA sequencing, we first construct gene libraries from *Bam*HI-, *Bgl*II-, *Pst*I-, *Xho*I-, *Hind*III-, *Eco*RI-, *Bcl*I-, *Cla*I- and *Alu*I-generated chloroplast DNA fragments using the plasmids pBR322, pKC7, pUC13, pUC18 and pUC19. These clones were used for DNA sequencing by the method of Maxam and Gilbert³⁰, and the dideoxy chain termination procedure³¹. About 95% of the sequence of each clone was obtained from both strands by the shotgun procedure³² and progressive deletion method³³, then the single-stranded region or ambiguities were resolved by the more directed enzymatic digestion method. We have sequenced both IR regions and found them to be identical. The data were compiled in computers using the program DNASIS (Hitachi SK Ltd.).



clover-leaf structure, except for tRNA^{Pro}_{GGG} in which the aminoacyl stem structure is incomplete. We identified 37 tRNA genes encoding 32 species, including 5 duplicated ones in the inverted repeat regions. The accepting amino acids were deduced from their unmodified anticodons according to the standard codon table¹¹. The tRNAs encoded by the chloroplast genome are sufficient to read all codons, taking into account wobbling and modification in the anticodons¹² and assuming that no tRNA is imported from the cytoplasm¹³. An analysis of codon usage in the ORFs reveals a preference for A or U in the third position of the codons.

Significant open reading frames were identified using the universal codon table (AUG as the initiation codon; UAA, UAG and UGA as termination codons). The introns in the ORFs were predicted from the presence of the 5' consensus sequence (GUGYG) and 3' consensus secondary structures characteristic of group II introns found in fungal mitochondrial genes⁵. These significant ORFs were identified by computer search¹⁴ using the protein sequence database NBRF PIR Release 6.0. The genes identified on the chloroplast genome are summarized in Table 1.

Chloroplasts contain their own ribosomes (70S) which are active in protein synthesis. About one-third of the ribosomal proteins are encoded by the chloroplast genome and the remainder by the nuclear genome¹⁵. We identified genes encoding ribosomal proteins by comparing amino-acid sequences with homologous genes in *Escherichia coli*. Moreover, there are clusters of *S12-S7* and *L23-L2-S19-L22-S3-L16-L14-S8-infA-secX-S11-rpoA* genes in a similar order to the clusters that have been reported in the *E. coli* ribosomal protein operons, that is, the *str* operon (*S12-S7-fus-tufA*) and a large cluster of ribosomal protein genes consisting of three operons, *S10* (*S10-L3-L4-L23-L2-S19-L22-S3-L16-L29-S17*)¹⁶-*spc* (*L14-L24-L5-S14-S8-L6-L18-S5-L30-secY-secX*)¹⁷-*α* and (*S13-S11-S4-rpoA-L17*)¹⁸, respectively; the genes found in chloroplast clusters are italicized. In contrast, the chloroplast genes *rps2*, *rps4*, *rps14*, *rps15*, *rps18*, *rpl20*, *rpl21* and *rpl33* were scattered throughout the

chloroplast genome¹⁹. We have reported previously that the coding sequence for the ribosomal protein S12 (*rps12*) is interrupted and located far apart on different DNA strands, suggesting the possibility of *trans*-splicing²⁰.

We also identified the genes *rpoA* and *rpoB*, which respectively encode the α - and β -subunits of *E. coli* RNA polymerase. The coding region for the β '-subunit consists of two separate ORFs (*rpoC1* and *rpoC2*, which correspond to the N-terminal and C-terminal halves of the β '-subunit, respectively), but this does not exclude the possibility of an intron lying between them (see Fig. 1). So far no gene has been detected corresponding to an *E. coli* type of σ factor²¹.

The most important feature of chloroplasts is their role in photosynthesis. Complete sequence analysis of the liverwort chloroplast DNA has revealed that there are nine genes (*psaA*, *psaB*, *psaC*, *psaD*, *psaE*, *psaF*, *psaG* and *psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF* and *psbG*) in photosystems I and II. We also identified the genes encoding six of the nine subunits of H⁺-ATPase (*atpA*, *atpB*, *atpC*, *atpD*, *atpE* and *atpF*). In addition, we have identified and mapped three genes (*petA*, *petB* and *petD*) encoding proteins involved in the photoelectron transport system. The gene for the large subunit of a ribulose biphosphate carboxylase (*rbcL*) is encoded by the chloroplast genome as expected, the small subunit being encoded by the nuclear genome. Our results revealed that the liverwort chloroplast genome is comparatively small (121,024 bp), nevertheless it possesses all the photosynthetic genes and others which have been reported previously in various plant species²², except the genes *tufA* (ref. 23) and *rps16* (ref. 24). These results imply that the chloroplast genomes in plants from liverwort to higher plants, have basically the same gene composition.

We have described a number of ORFs which have not been reported or identified previously. Our computer analysis revealed seven ORFs (*ndh1*, *ndh2*, *ndh3*, *ndh4*, *ndh4L*, *ndh5* and URF6) whose amino acid sequences show significant homology (11.3–31.3% over their entire length, but much higher in specific

Table 1 Genes encoded by the chloroplast DNA from a liverwort, *M. polymorpha*

rRNA genes (IR _A and IR _B) 16S, 23S, 4.5S and 5S	Genes for photosynthesis	Genes predicted by amino-acid sequence homology
tRNA genes 37 tRNAs (see Fig. 1)	<i>rbcl</i> , large subunit of ribulose biphosphate carboxylase	<i>ndh1*</i> , homologous to mammalian mitochondrial URF1
RNA polymerase genes <i>rpoA</i> : homologous to <i>E. coli</i> α -subunit <i>rpoB</i> : homologous to <i>E. coli</i> β -subunit <i>rpoC1*</i> : homologous to <i>E. coli</i> β' -subunit <i>rpoC2</i> : homologous to <i>E. coli</i> β' -subunit	<i>psaA</i> , photosystem I P ₇₀₀ chlorophyll <i>a</i> apoprotein <i>psaB</i> (same as above) <i>psbA</i> , photosystem II 32K protein <i>psbB</i> , photosystem II P ₆₈₀ chlorophyll <i>a</i> apoprotein	<i>ndh2*</i> , URF2 (same as above) <i>ndh3</i> , URF3 (same as above) <i>ndh4</i> , URF4 (same as above) <i>ndh4L</i> , URF4L (same as above) <i>ndh5</i> , URF5 (same as above)
Ribosomal protein genes and related genes 50S subunit <i>rpl2*</i> , <i>rpl14</i> , <i>rpl16*</i> , <i>rpl20</i> , <i>rpl21</i> , <i>rpl22</i> , <i>rpl23</i> , <i>rpl33</i> 30S subunit <i>rps2</i> , <i>rps3</i> , <i>rps4</i> , <i>rps7</i> , <i>rps8</i> , <i>rps11</i> , <i>rps12*</i> , <i>rps14</i> , <i>rps15</i> , <i>rps18</i> , <i>rps19</i>	<i>psbC</i> (same as above) <i>psbD</i> , photosystem II D2 protein <i>psbE</i> , cytochrome <i>b</i> ₅₅₉ <i>psbF</i> (same as above) <i>psbG</i> , photosystem II G protein <i>atpA</i> , ATPase F ₁ subunit α <i>atpB</i> , ATPase F ₁ subunit β <i>atpE</i> , ATPase F ₁ subunit ϵ <i>atpF*</i> , ATPase F ₀ subunit I <i>atpH</i> ATPase F ₀ subunit III <i>atpI</i> , ATPase F ₀ subunit IV <i>petA</i> , cytochrome <i>f</i> <i>petB*</i> , cytochrome <i>b</i> ₆ <i>petD*</i> , subunit 4 of cytochrome <i>b</i> ₆ / <i>f</i> complex	URF6, homologous to <i>Aspergillus nidulans</i> mitochondrial URF6 (human mitochondrial URF6) <i>frxA</i> , homologous to 4Fe-4S type ferredoxin <i>frxB</i> (same as above) <i>frxC</i> , homologous to 4Fe-4S protein found in <i>R. capsulata</i> <i>mhpX</i> , homologous to ATP-binding subunit of inner membrane permease in <i>E. coli</i> (<i>malK</i>) or <i>Salmonella typhimurium</i> (<i>hisP</i>) ²⁹
Others <i>infA</i> , <i>secX</i>		Unidentified genes More than 28 ORFs

Abbreviations for genes are the same as in Fig. 1

* Indicates the presence of introns in the coding sequences.

regions) to those of human mitochondrial genes (URFs)⁶. Six of the ORFs (URFs 1-5 and 4L) were identified as genes for components of the NADH dehydrogenase in human mitochondria⁷. There has been no previous report of the presence of these genes in the chloroplast genome. However, an NADH-plastoquinone-(PQ) oxidoreductase activity has been detected in the chloroplasts of *Chlamydomonas reinhardtii*²⁵, thus it is possible that these ORFs encode subunits of the NADH-PQ oxidoreductase. There are two cysteine-rich ORFs (*frxA* and *frxB*) in which the periodic appearance of cysteines is typical of that in 4Fe-4S ferredoxin²⁶, and an ORF (*frxC*) whose amino-acid sequence is highly homologous (44.3%) to that of the ORF in the photosynthetic gene cluster of *Rhodospseudomonas capsulata*²⁷. These findings imply that basic genes which encode components of the large subunit of ribulose biphosphate carboxylase photosystem I, H⁺-ATPase, the cytochrome *b*₆/*f* complex, photosystem II and NADH-PQ oxidoreductase, all reside on the chloroplast genome. These functionally related gene groups are to some extent clustered in the chloroplast genome (see Fig. 1).

Thus, the liverwort chloroplast genome contains genes encoding the transcription-translation system as well as genes for protein complexes involved in chloroplast photosynthesis.

However, it seems that no functionally active complex is formed without nuclear-encoded protein subunits. Strikingly, chloroplasts and mitochondria have been found to have genes with a common function, in particular, the genes encoding H⁺-ATPase, NADH dehydrogenase and the cytochrome complex²⁸. These observations can be interpreted as suggesting that chloroplasts and mitochondria arose from a common ancestor. Further detailed information obtained from the complete sequencing of liverwort chloroplast DNA will be published elsewhere (but liverwort chloroplast DNA sequence data are available on request).

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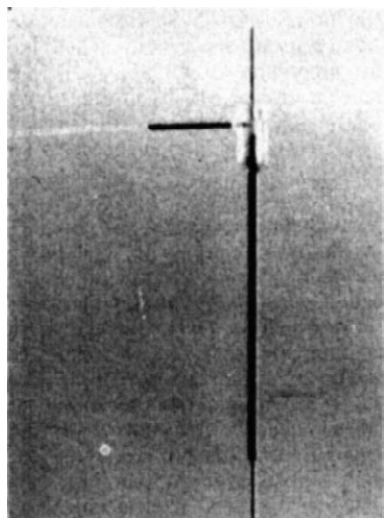
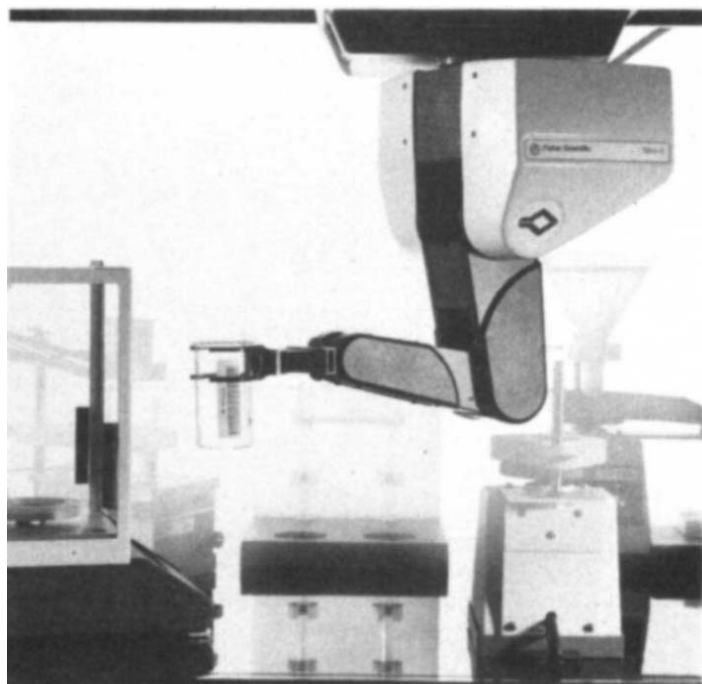
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Tradition takes an unexpected turn

New equipment and methodology keep breaking old moulds. This week an all-purpose robot rides on the ceiling, mussels make tissue culture a sticky situation and a fungus fights the planthopper that feeds it.

A microdialysis probe that acts as an "exogenous blood vessel" could help monitor the activities of neurotransmitters and the effects of neurotoxins (Reader Service No. 100). The probe represents the combined efforts of Carnegie Medicine in Sweden and a research team at the Karolinska Institute, who sought to find a technique that could dynamically follow chemical events in living tissue. Using two concentric steel cannulas tipped with a dialysing membrane, the collaborators found that substances diffuse across the membrane when a physiological fluid enters through the inner cannula, flushes the inside of the membrane and exits through the outer cannula. Hence Carnegie's probe can recover endogenous substances without removing fluid and introduce exogenous substances without injecting liquid. Although the concept originated with studies of the brain, the company now supplies probes for different purposes and locations within the body. An

Fisher keeps its robot in suspense, but the Maxx-5 can work on solid ground as well. The robot runs on an IBM PC that can manage both the robot and the data it collects.



A simple solution for complex studies.

assortment of membranes is also available with molecular weight cut-offs between 5,000 and 50,000. Complete instrumentation for microdialysis includes the probe, a pump and a liquid switch, but Carnegie also manufactures tools for subsequent fraction analysis by liquid chromatography.

Low overhead

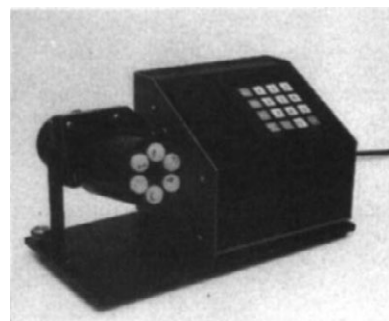
It weighs 30 lb, runs by computer and costs between \$30,000 and \$50,000 (US) and it won't get in the way (Reader Service No. 101). Fisher's Maxx-5 robot can be mounted on laboratory ceilings or overhead frames using a motorized track that

can propel it along at a foot per second. The track, which also mounts on benches, adds a sixth dimension to Maxx-5's five-axis articulated arm. With adjustable grip strength, programmable arm speed and the aid of an IBM PC, the robot will dilute, mix, centrifuge, pipette, filter, concentrate and separate samples. Its fingers can hold beakers with up to 800 ml of solution. Maxx-5 also reads bar coding, so it can process samples according to individual labelling rather than by relying on station position. At this year's Pittsburgh Conference, the company said that Maxx-5 drew 570 inquiries. Fisher has a brochure entitled "Partners in Productivity" that describes the system in greater detail. The personal computer, software, linear track and numerous appliances are available as accessories.

Making the gradient

High reproducibility and convenience have rarely been the hallmarks of sucrose gradient generators. But a fresh approach from a professor at Canada's University of New Brunswick may turn gradient making around (Reader Service No. 102). According to inventor David Coombs, BioComp's model 105 will generate six identical sucrose gradients in 2.5 min without requiring any user supervision. Tubes that have been layered half and half with heavy and light sucrose are put in the instrument. It then tilts them to a predeter-

mined angle and rotates them for a pre-set time, at a pre-set speed. Different and predictable gradient distributions result from different combinations of angle, time and speed. These parameters can be stored in the 105's non-volatile computer memory. Interchangeable holders will accommodate most tube sizes. At about \$1,900



An innovation in reproducible gradients.

(US), Coombs says that the model is "more expensive, but less trouble" than traditional two-chamber devices.

Mussel power

The same adhesive that keeps the common blue mussel anchored to rocks and piers can now be used to attach cells and tissue to glass, plastic and even Teflon (Reader Service No. 103). Connecticut based BioPolymers Inc supplies the pro-



Skeletal imposters

THIS PRISTINE form belies its origin in oil and sediments far beneath the Earth's surface (Reader Service No. 104). The crystal line (18βH)-olean-12-ene-3-one pictured is just one of the many substances that clutter petrochemical and geochemical analyses. In the search for oil in particular, reliable internal standards for analysis equipment are necessary to ensure the authentic identification of deposits. Recently, high purity "biomarkers" have emerged in the fossil fuel industry as dependable reference sources. These markers mimic the carbon skeletons of the organisms from which oil deposits are formed. By calibrating gas chromatography and mass spectrometry equipment with these synthetic markers, the distribution, history and progressive transformation into fossil fuel of the genuine organisms can be "unearthed".

tein, which it calls Cell-Tak, in 2 mg quantities of sterile aqueous solution for \$90 (US). The company claims Cell-Tak works rapidly with virtually any substrate; typically greater than 80 per cent of cells attach within 10 min of seeding, suggesting a diffusion-limited process. The adhesive does not alter cell morphology or doubling time and its effects appear to be independent of cell type. BioPolymers has already tested its product with anchorage-dependent cell lines such as vascular endothelium, as well as anchorage-independent lines such as U-937 human lymphoma. The company must shell, by hand, about 3 million mussels to extract 1 lb of Cell-Tak adhesive. Thankfully, it takes only 50 μl of Cell-Tak to coat a 35 ml Petri dish.

Instant access

The British Bureau of Hygiene and Tropical Diseases now provides several comprehensive **current awareness services on AIDS** and retroviruses (Reader Service No. 105). The bureau's update service publishes monthly an annotated bibliography of all the papers and articles on

AIDS that the bureau has located, grouped by subject category. The update subscription rate is £80 (UK) or \$160 (US). Selected papers on AIDS and retroviruses also make it into one of the bureau's two abstract journals, and an AIDS newsletter has been established to cover the main AIDS news stories and commentaries on scientific developments. The BHTD recently made both the update and the abstract journals available for on-line searching through CAB International. These services are also on DataStar and BRS Information Technologies.

A **free literature search** from Radiometer Analytical can bring a wealth of experience as close as the telephone (Reader Service No. 106). Radiometer compiled selections from its applications library into a computer data base, organised by key words such as ion species or sample types. RADABASE now contains more than 1,500 references on Radiometer equipment use and misuse. The company supplies hard copies of the search for every request.

Small wonder

A **cell disrupter for small samples** uses minute glass beads and violent agitation to accomplish its ends (Reader Service No. 107). The Mini-BeadBeater, a recent release from Biospec Products in Oklahoma, uses a sealed polypropylene chamber within which 1 ml of beads will disrupt about 1 ml of sample in 1 to 3 min. Biospec

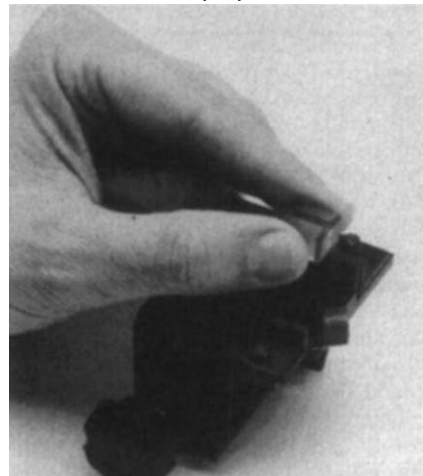


Small-scale destruction from Biospec.

says that the collisions of the glass beads will crush even tough samples, like bone or microbial spores, without foaming or aerosol formation. The company thinks its system is safe for isolating enzymes and organelles or for extracting nucleic acids by phenol/chloroform procedures. In addition to applications with biological materials, Biospec recommends the Mini-BeadBeater for wet milling and emulsifying non-biologicals such as soil, paint pigments and pharmaceutical samples. With 15 disposable vials, two bead sizes optimized for eukaryotic or bacterial disruption and a 0.62 amp motor, the instrument sells for \$365 (US).

Beckman has tilted its latest **spectrophotometry system** towards the nucleic

acid researcher, with a design that accommodates sample volumes of less than 50 μl (Reader Service No. 108). The company says its Micro-Leader package gives repeatable measurements and virtually full recovery that simplify purity analyses in DNA preparation. Beckman



Beckman's spectrophotometer is made for minute samples.

credits this performance to the micro-volume cuvette and cell holder that it designed specifically for the Micro-Leader system. With this cuvette samples are inserted and removed with a micropipette or pasteur pipette. The full \$8,800 (US) system includes a DU-50 spectrophotometer and a software applications module with five procedures for purity assessment by ratio methods. These programs, says Beckman, reduce wavelength selection, measurement and calculation to a few key strokes.

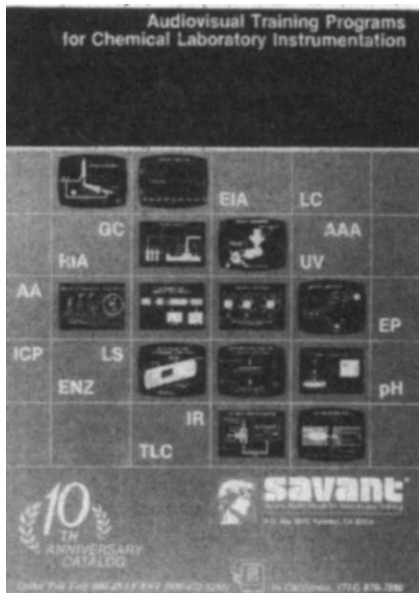
Courses of action

Hands-on experience with sophisticated techniques can be a rarity for biology undergraduates, so a Purdue professor decided to pull together the materials and literature needed to teach them electrophoresis (Reader Service No. 109). Today the **electrophoresis educational kits** are marketed by Indiana-based Biostar Corp. Biostar adapted research procedures for use in student laboratories, and now even high schools are taking advantage of the opportunity — they comprise 25 per cent of sales. The kits are divided into three different levels of user experience and each provides the chemicals, equipment and manuals for about 16 students. Biostar considers its product a helpful boost into a biotechnology career.

Time for quick decisions: prices are about to go up on LC Resources' five-hour **video primer for HPLC** (Reader Service No. 110). The course, entitled "Getting Started in HPLC", covers the basic aspects of the chromatography method in five modules that can be purchased separately or as a \$1,795 (US) package. Pricing may limit the feasibility of the course to group training.

and LC Resources expects to raise its price to \$2,250 (US) in September. But the company guarantees the programme with complete "credit" (presumably not a refund) on returns within 30 days, and negotiates discounts for multiple orders. Video tapes come in 1/2-inch VHS and 3/4-inch U-Matic formats.

Savant's 10th anniversary catalogue gives a run-down of current **training programmes** in analytical, clinical and chemical instrumentation, along with three of the California company's latest additions (Reader Service No. 111). The new programmes include: training for inductively coupled plasma MS, enzyme immunoassays and gas chromatography analysis. The latter two programmes are authored by William Ulrich at Smith-Kline Beckman and Virginia Polytechnic's Harold McNair, respectively. Savant provides its training courses as slides or video tapes. The slide packages contain about 50 slides, a script and a cassette tape; the videos are available in VHS



Savant trains all types.

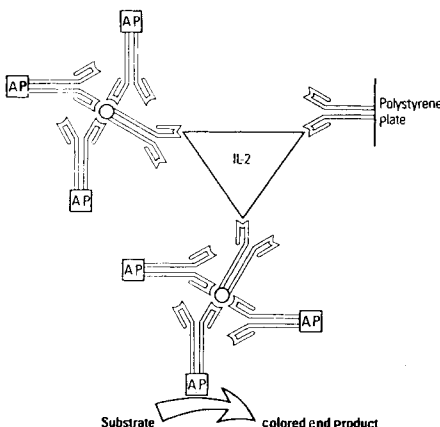
or Beta formats. Savant says the courses last about 45 minutes, and prices are listed in the catalogue.

The AIDS brigade

Earlier this summer, Du Pont announced the arrival of a **radioimmunoassay** that directly detects a structural core protein of the AIDS virus, HTLV-III (Reader Service No. 112). Du Pont says that its method is 25 to 200 times more sensitive than reverse transcriptase assaying and cuts cell culturing time down to as few as 3 days. The kit, which is at present available for research purposes only, measures viral activity as represented by the amount of p24, a core protein. According to the company it "virtually eliminates cross reactivity with other human cellular and viral proteins, and has been able to detect all human AIDS-associated retroviruses tested to date". Du Pont includes

recombinant human interleukin-2 in the kit to amplify cell growth in culture. Assays can be performed in less than a day. Du Pont charges \$650 (US) for the kit, which contains enough material for 125 assays.

A scheme for detecting human interleukin-2 using monoclonal antibodies hit the market recently when Genzyme Corp in



The final step in Genzyme's analysis.

Boston introduced its InterTest-2 kit (Reader Service No. 113). Taking less than 8 hours to complete, the ELISA-based technique is distributed for research use only and can detect IL-2 in serum, blood and other biolo-

gical fluids, says Genzyme. The company collaborated with a Boston group called Endogen to produce the kit, and expects the assay to speed IL-2 research into cancer and AIDS treatment. Genzyme sells InterTest-2 kits for \$395 (US) each, and each kit can perform up to 84 tests.

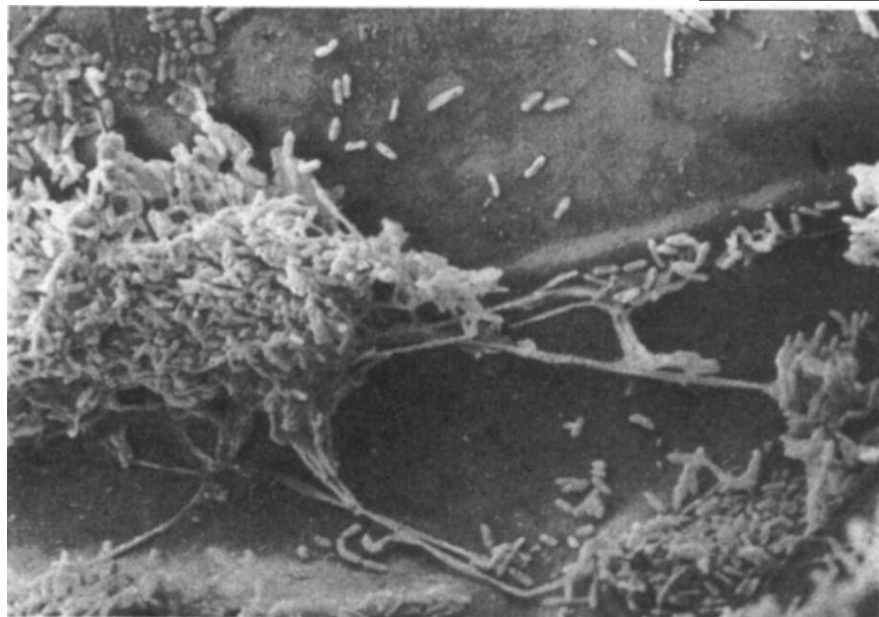
Dealing with DNA

Applied Biosystems keep experimenting with the chemistry for their **DNA synthesizers**, and they are now producing strands up to 175 bases long (Reader Service No. 114). AB's model 380B synthesizer is one of a family of machines capable of this sort of



New chemistry boosts Applied Biosystems' synthesizer.

production. Using β -cyanoethyl phosphoramidite methods, the 380B routinely gener-



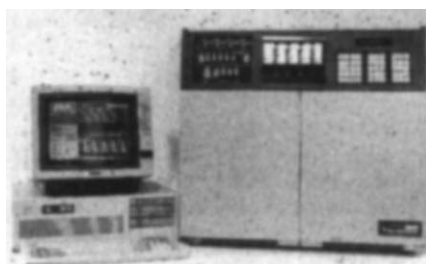
Spore wars

THIS SEEMINGLY sedate micrograph actually depicts a vicious attack by the green fungus *Metarhizium anisopliae* upon the brown planthopper *Nilaparvata lugens* (Reader Service No. 115). Three researchers at King's College, London and the Berkshire company Microbial Resources plan to make *M. anisopliae* even more adept at victimizing the planthopper, which causes serious losses to rice crops in China, Japan, Korea and India. The investigators want to exploit the

microbe's role as a natural pesticide. Using techniques for genetic recombination, such as somatic hybridization and traditional heterokaryon fusion, they hope to increase the fungus's effectiveness in the field. Already the team has isolated one recombinant that gives nearly twice the total spore production and over 1,000 times the ease of spore release of either parent. These improvements have so far been achieved without any adverse effects on *M. anisopliae*'s growth rate.

ates 50- to 100-mers and quantities that range from primers and probes up to 10 mg for physical studies. Coupling yields vary between 98 and 100 per cent, according to AB. A single-column instrument costs \$45,000 (US) and a 5-column, \$49,500 (US). A menu-driven touchscreen control allows the user to operate standard synthesis cycles including automatic deprotection and cleavage), or develop new procedures.

Two new synthesizers from Biosearch in California have also been making their mark (Reader Service No. 116). The models 8650 and 8750 have one and four columns, respectively, and according to Biosearch have been producing DNA fragments up to 180 bases in length. An IBM PC/AT computer workstation manages the unit, stores sequence and protocol data and simplifies sequence entry and editing. Biosearch mentions that the computer can also be connected via a modem to libraries of DNA



Biosearch is joining the synthesizer league.

sequences and to software that could translate sequences into protein equivalents. The IBM will control up to eight of Biosearch's instruments, which cost \$42,900 and \$53,900 (US), depending on the number of columns, and use β -cyanoethyl phosphoramidite chemistry.

Proteins A and G

An alternative to protein A as an immunological reagent has come from Amersham in the form of protein G, a bacterial cell wall protein with a high affinity for the Fc region of a range of immunoglobulins (Reader Service No. 117). Amersham lists among protein G's advantages high affinity binding to IgGs from rat, goat and sheep and binding almost exclusive to IgG without cross-reactions with other classes. The reagent, according to Amersham, also binds to a wider range of species IgG and subclasses than protein A. But the company suggests

that ultimately its 125 I-labelled protein G would best be viewed as a complement to protein A. As such, it could be particularly useful in blotting procedures to detect specific IgGs, in the study of antigen-antibody interactions and for antibody screening where protein A fails to bind.

Two fusion vectors and a revised Sepharose gel put the punch into Pharmacia's system for the production, purification and characterization of **protein A fusion protein conjugates** (Reader Service No. 118). The company's fusion system uses the two vectors to induce high levels of expression of intracellular proteins (vector pRIT2T) and of proteins transported to the periplasmic space or secreted (pRIT5). According to Pharmacia, conjugates thus produced in prokaryotic expression systems can be purified in a single step owing to improved mechanical characteristics of IgG Sepharose 6 gel. The company points out that the repetitive structure of the protein A moiety may enhance the immune response, making conjugates well-suited for direct immunization. Immunological assays will also provide rapid detection due to the protein A tail.

Good references

A simple test for pipettors can provide reassurance when the instruments are accurate and warning when they go on the blink (Reader Service No. 119). Bel-Art Products supplies an accuracy test kit that determines whether pipettes from Gilson, Eppendorf, Oxford, Finn and other manufacturers still conform to those brands' specifications for precision. An adaptor slips into the pipettor tip, then a capillary is inserted in the adaptor's distal end. Next the pipettor plunger is depressed, the capillary immersed in water and the plunger slowly released. The liquid level in the capillary should be within 1.5 mm of Bel-Art's mark on the glass; otherwise, the pipettor is not performing within manufacturer's specifications. The \$87 (US) kit has two soft adaptors and six different sizes of glass inserts to adjust for volumes between 1 and 1,000 μ l.

A set of nine mercury-filled **thermometers** may look ordinary but carry a hidden value: Brooklyn Thermometer Co's reference set has NBS traceable certification (Reader Service No. 120). The yellowback glass thermometers cover the temperature range between -36° and 761° F. Brooklyn guarantees fractional degree accuracy for one year and charges \$1,124 (US). The set comes with factory certificates and corrections for the five text points on each thermometer, as required by the American Society for Testing and Materials. ☐

These notes are compiled by Karen Wright from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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Amersham's protein G may beat protein A binding

Ig	Bacterial Ig I <i>S. aureus</i> (Protein A)	Fc receptor type III Group C and <i>G. streptococci</i> (Protein G)
Human IgG1	■	■
Human IgG2	■	■
Human IgG2	□	■
Human IgG4	■	■
Mouse IgG1	☒	☒
Mouse IgG2a	■	■
Mouse IgG2b	■	■
Mouse IgG3	■	■
Rat IgG1	☒	☒
Rat IgG2a	□	■
Rat IgG2b	□	☒
Rat IgG2c	■	■
Rabbit IgG	■	■
Bovine IgG1	□	■
Bovine IgG2	■	■
Sheep IgG1	□	■
Sheep IgG2	■	■
Goat IgG1	☒	■
Goat IgG2	■	■
Horse IgG(ab)	☒	■
Horse IgG(c)	☒	■
Horse IgG(T)	□	☒

- ☐ No reaction
☒ Weak reaction
☒ Strong reaction

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